

**Post-Translational Modification and Regulation of the Chaperone
Hsp27 during Apoptosis**

A Senior Honors Thesis

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CHAPTER 1

INTRODUCTION

1.1 Apoptosis

Apoptosis, or programmed cell death (PCD), is an essential mechanism for the regulation of cellular homeostasis and normal development. Apoptosis requires precise regulatory and signaling pathways to orchestrate the timing and progression of cell death. Among the processes dependent upon apoptosis are organ formation and regulation of the immune system, as well as successful completion of embryo development (1, 2). Apoptosis is characterized by various biochemical and morphological changes, including DNA fragmentation, plasma membrane blebbing, and the externalization of signaling molecules such as phosphatidylserine, which lead to the formation of apoptotic bodies (3). Of particular importance is the deregulation of apoptosis. The failure of a cell to undergo apoptosis is central to cancer progression and chronic inflammatory diseases. On the contrary, uncontrolled apoptosis may lead to neurodegenerative diseases such as Parkinson's and Alzheimer's (4-6).

Apoptosis is executed by a family of conserved cysteine proteases called caspases. Caspases involved in apoptosis are characterized into two groups, the initiator caspases, such as caspase-8 and caspase-9, and the effector caspases, such as caspase-3 and caspase-7. All caspases are synthesized in cells as inactive precursors called pro-caspases that become activated by various apoptotic stimuli (7-9). Activation is induced through a series of proteolytic cleavages that result in an assembly of tetramers. The activation of an effector caspase is performed by an initiator caspase through an internal

cleavage to separate the large and small subunits. The activation of an initiator caspase, however, is autoactivated under apoptotic conditions and usually facilitated by large multicomponent complexes often called apoptosomes (10). The active enzymes are heterotetramers composed of two identical subunits of ~20 kDa and two identical subunits of ~10 kDa, whereas the prodomain or amino-terminal domain is usually cleaved during activation (11). Initiator caspases typically contain an extended N-terminal prodomain that is important for protein-protein interactions. In contrast, effector caspases typically contain a shortened prodomain often implicated in binding to regulatory proteins (12). All known caspases possess an active-site cysteine, and cleave substrates after aspartic residues at Asp-Xxx bonds; the distinct substrate specificity of caspases is determined by the four residues amino-terminal to the cleavage site (13). Cleavage induces activation or inactivation of substrates that results in the characteristic biochemical changes that progress cells towards death through the formation of apoptotic bodies (3).

Two main pathways exist for caspase activation: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway, or the receptor mediated-pathway, involves the binding of apoptotic stimuli to a receptor, such as the Fas receptor, that contains a cytoplasmic death domain essential for transduction of the apoptotic stimuli. Receptor-ligand association promotes the formation of an intracellular complex of adaptor proteins including FADD (Fas-associated death domain) which triggers the recruitment of pro-caspase-8 and the subsequent activation of the caspase cascade (14, 15). In contrast, the intrinsic pathway is receptor-independent and is commonly initiated by mitochondrial dysfunction. Apoptotic stimuli, such as cytotoxic drugs, produce changes in the

mitochondrial membrane potential which allows for the leakage of pro-apoptotic molecules such as cytochrome c, into the cytoplasm, promoting the activation of caspase-9. Released cytochrome c binds to Apaf-1 and the cofactor dATP, resulting in the formation of the apoptosome. Together, these molecules allow for the activation of caspase-9 (16). The activation of caspase-9 can also be accomplished through the receptor-mediated pathway following the activation of caspase-8 (17) serving as a means of communication between the intrinsic and extrinsic apoptotic pathways. In addition to cytochrome c, radical oxygen species (ROS) are often released from the mitochondria due to disruption of the electron transport chain (18) and contribute to the activation of the apoptotic program (19). Regardless of the pathway, however, signaling cascades converge on the activation of downstream effector caspases such as caspase-3, -6, and -7 (20), which are collectively responsible for the cleavage of target proteins and the subsequent biochemical changes accompanying apoptosis (21-23).

In addition to caspase activation, cell response to apoptotic-inducing drugs has been associated with the activation of apoptotic kinases and the inactivation of survival kinases. The mitogen-activated protein kinase family (MAPK), which includes p38, JNK (Jun-N-terminal kinase), and ERK (extracellular signal-regulating kinase), has been implicated in cell proliferation, cell survival, and stress response (24). For instance, activation of p38 is associated with the induction of apoptosis in response to UV treatment and treatment with chemotherapeutic drugs (25, 26). By contrast, ERK can exhibit either anti-apoptotic or pro-apoptotic functions depending on the stimuli and cell type. In monocytes, ERK phosphorylation has been shown to inhibit caspase-9 activation and therefore apoptosis (27). However, up-regulation of ERK is associated with

apoptosis, especially during treatment with flavonoids (28). In addition to MAPKs, members of the protein kinase C family (PKC) have been shown to regulate cell death. In particular, PKC δ has been suggested as a pro-apoptotic molecule as PKC δ activation has been associated with cell death in various cell types in response to a variety of apoptotic stimuli (29). For instance, PKC δ has been shown to phosphorylate and activate caspase-3 (30). PKC δ has also been identified as a substrate of caspase-3 and its proteolytic cleavage results in a constitutively active catalytic domain necessary for apoptosis (31, 32). Additionally, PKC δ has been shown to translocate to the mitochondria and alter its membrane potential (33) and promote ROS production (34, 35).

1.2 Flavonoids

Increasing attention has been focused on the use of compounds derived from fruits and vegetables for epidemiological purposes such as cancer prevention and oxidative stress protection. The principal plant-derived agents thought to provide cancer prevention and antioxidant properties are flavonoids, which are the most common and widely distributed polyphenolic compounds, ubiquitously present in foods of plant origins (36). Flavonoids are composed of a common phenyl chromanone structure with one or more hydroxyl substitutes (Fig 1.1). Depending on the organization of their benzene rings and their modifications, flavonoids are generally classified into flavones, flavanols, isoflavones, flavanols, flavanones, and anthocyanins (36, 37). Flavonoids have long been recognized as having anti-cancer, anti-inflammatory, anti-viral, anti-bacterial, and anti-allergic properties (38-40). For example, the flavanone naringenin, which is found abundantly in grapefruits, has been shown to have an anti-

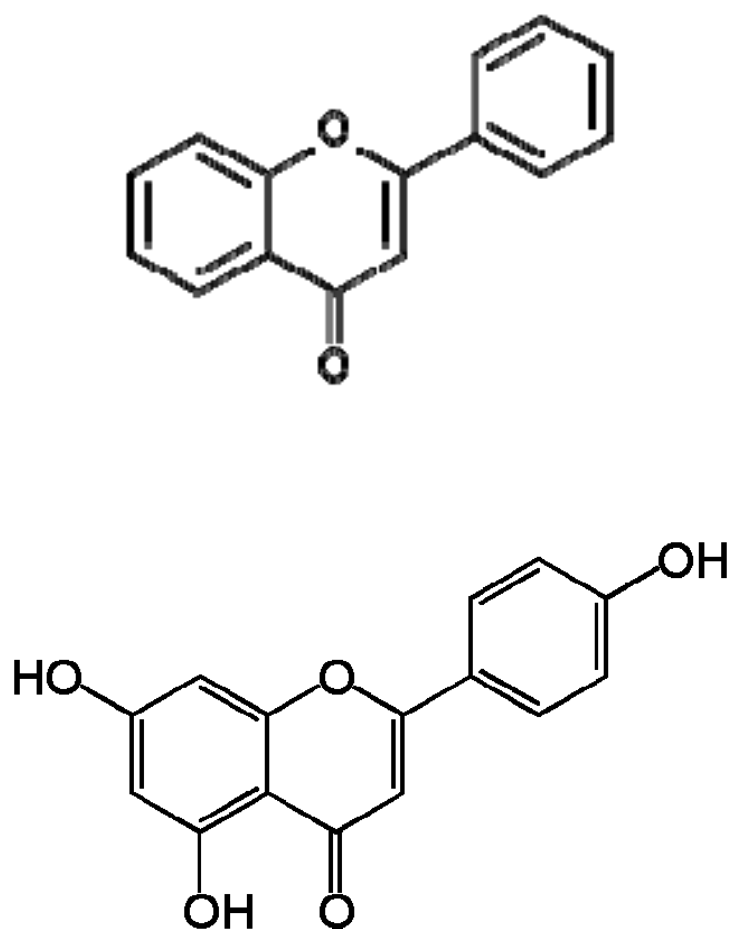


Figure 1.1 Structure of typical flavonoid (top) and apigenin (bottom)

carcinogenic effect in breast cancer (41). The flavone apigenin, which is found abundantly in broccoli, parsley, and chamomile tea, has been reported to induce apoptosis in breast cancer, colon cancer and lymphocytic leukemia (42-44). In particular, apigenin has been shown to induce mitochondrial depolarization and the production of reactive oxygen species which allows for the release of cytochrome c and procaspase-9 from the mitochondria and the induction of the caspase-dependent apoptotic pathway (44-46). Recently, a role for apigenin in monocytic leukemia cells was identified. It was shown that apigenin localizes to the mitochondria of leukemia cells and stimulates the apoptotic pathway in a PKC δ and caspase-dependent mechanism. However, in contrast to many other cell types, ROS production and MAPK activation were shown to act independently of apoptosis induction (47). In addition, there is very little evidence to suggest that apigenin produces adverse metabolic effects when consumed in nutritionally relevant quantities. In fact, it has been shown that apigenin reduces proliferative properties of leukemia cells at a much greater capacity than the flavonoid's effect on the epithelial cells MCF7 and A549 (47). Therefore, the use of apigenin as a chemopreventive agent may provide a tissue-specific defense against cancer and oxidative stress.

1.3 The small heat shock protein Hsp27

The small heat shock protein 27 (Hsp27) belongs to a family of molecular chaperones that have recently been focused on as regulators of cell death and survival. Hsps encompass several groups of proteins and are classified by size, structure, and function: Hsp110, Hsp90, Hsp70, Hsp60, and the small Hsp families (48). Some Hsps are constitutively expressed and increase in response to environmental and physiological

stress. In unstressed cells, Hsps are essential for maintaining cellular homeostasis, functioning as molecular chaperones to help facilitate protein folding (49-51).

The small heat shock proteins (sHsps) are low molecular mass chaperones ubiquitously present throughout all kingdoms. The sHsps are ATP-independent chaperones characterized by a conserved crystallin domain flanked by variable N- and C-terminal regions (52, 53). In unstressed cells, sHsps exist as large oligomeric complexes whose oligomerization state is dependent on the degree of phosphorylation of the individual monomers. Phosphorylation at the N-terminus results in a redistribution of the large oligomer into smaller tetramer units whereas dephosphorylation promotes the formation of large oligomers (54, 55). It is thought that the chaperone activity of sHsps is correlated with this dynamic oligomerization. Exposure to stress promotes the formation of misfolded proteins that may bind large non-phosphorylated sHsp oligomers or smaller complexes such as dimers to prevent aggregation. Upon return to favorable conditions, these proteins are released and either refold spontaneously or with the help of the ATP-dependent chaperones such as Hsp70 (56).

More recently, focus has shifted towards the potential anti-apoptotic functions of the sHsps, with a particular emphasis on Hsp27. Hsp27 is known to block apoptosis at different stages due to its interaction with other proteins. As a molecular chaperone, Hsp27 plays an important role in the prevention of protein aggregates following misfolding. Sufficiently large amounts of misfolded proteins can result in the formation of these aggregates, which can signal for the induction of apoptosis (50, 51). Hsp27 can also protect against protein aggregation at the pre-translational level by binding to the translation initiation factor eIF4G and preventing the translation of mRNAs under

stressful conditions, a common cause of protein misfolding (49). Additionally, Hsp27 has been shown to protect against apoptosis by regulating mitochondrial dysfunction through inhibition of ROS production and mitochondrial membrane depolarization (34, 57-59). However, Hsp27 primarily exhibits its anti-apoptotic effect through direct interaction with several critical components of the apoptotic pathway (Fig 1.2). Following extrinsic stimuli, Hsp27 has been shown to exhibit its anti-apoptotic effect in a mitochondrial and caspase-independent manner by blocking the interaction of Daxx with the Fas death receptor (60). More importantly, Hsp27 functions as a negative regulator of the leakage of pro-apoptotic molecules normally residing in the mitochondria, which can result in caspase-3-dependent apoptosis. Hsp27 inhibits caspase-3 activation by sequestering pro-caspase-3 and cytochrome c (61), negatively regulating the activation of the pro-caspase-9 through interaction with cytochrome c (62), and inhibiting the activation of the pro-apoptotic Bax through a PI3K-dependent mechanism (63). Hsp27 has also been shown to regulate apoptosis by binding to the prodomain of caspase-3, inhibiting its activation (64). Thus, Hsp27 exhibits a robust anti-apoptotic activity with many members of the apoptotic pathway and many potential points of regulation.

It is recognized that interaction of Hsp27 with other proteins is regulated by the oligomerization and phosphorylation status of the protein. It has been suggested that large non-phosphorylated oligomers of Hsp27 are required for its caspase-dependent anti-apoptotic activity (65). Moreover, it has been shown that phosphorylation is responsible for the release of Hsp27 from its target proteins through dissociation into smaller multimers (55, 66). Hsp27 is phosphorylated at three different serine residues, Ser-15, Ser-78, and Ser-82, by mitogen-activated protein kinase-activated protein kinase 2 (MK2)

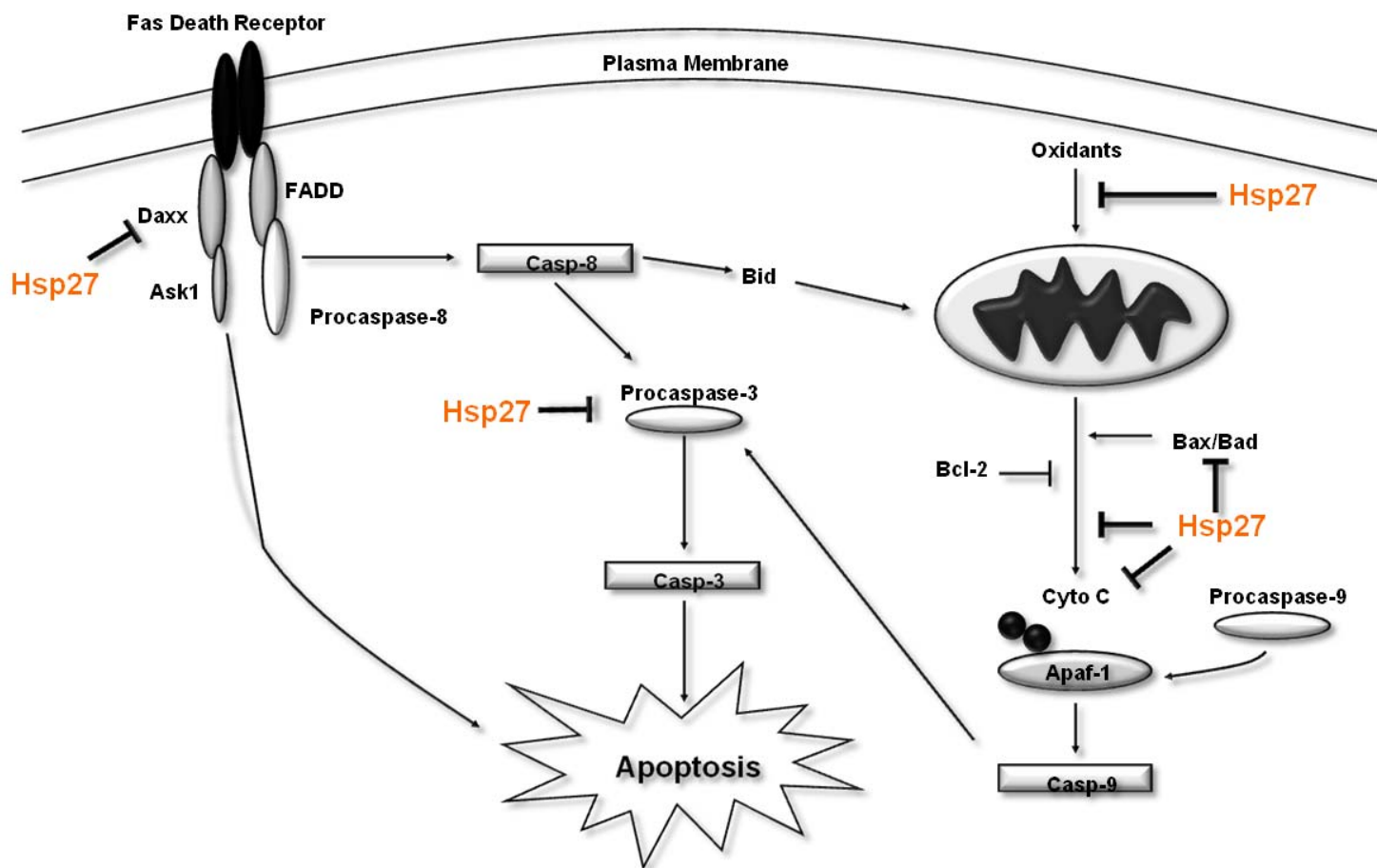


Figure 1.2 Anti-Apoptotic functions of Hsp27

Hsp27 can regulate apoptosis various points. Following extrinsic activation of the apoptotic pathway, Hsp27 can block Daxx-mediated apoptosis by preventing the translocation of Daxx to the plasma membrane and its association with the Fas receptor. Hsp27 also acts as a negative regulator of the leakage of pro-apoptotic molecules from the mitochondria by maintaining intracellular redox homeostasis and mitochondrial stability. Following stress-induced release of pro-apoptotic molecules from the mitochondria, Hsp27 can inhibit Bax in a PI3K-dependent mechanism and sequester cytochrome c release from the mitochondria. In addition, Hsp27 can regulate caspase activation both by preventing caspase-9 activation by association with cytochrome c and by binding to the prodomain of caspase-3.

as a result of p38 MAPK pathway activation (67, 68). Additionally, it has been found that PKC δ may act as an upstream kinase of p38, inducing the phosphorylation of Hsp27 by MK2 (69), or as the kinase directly responsible for Hsp27 phosphorylation (66, 70). Although p38 has been identified as the primary upstream kinase involved in MK2 activation, other families of MAPKs may play a role in Hsp27 phosphorylation. In particular, ERK has been identified as both a kinase responsible for MK2 phosphorylation (71) and a substrate of PKC δ (72). Therefore, the kinases involved in Hsp27 phosphorylation may exhibit both cell and stimulus specificity.

In this work, we sought to determine the regulators of Hsp27 phosphorylation during apigenin-induced apoptosis. Additionally, we sought to determine the role of Hsp27 phosphorylation in the chaperone's ability to protect against mitochondrial membrane depolarization. We first demonstrated that Hsp27 exhibits a bimodal phosphorylation during apigenin-induced apoptosis. We found that following a 15 min apigenin treatment, Hsp27 phosphorylation is p38 dependent. Additionally, we found that at 6 h post-apigenin treatment, Hsp27 phosphorylation at both Ser-78 and Ser-82 is p38 dependent. Moreover, we found that at 6 h, only Ser-82 is PKC δ dependent and full-length PKC δ can directly and specifically phosphorylate Hsp27 at Ser82. Furthermore, we found that p38 may act upstream of PKC δ during this phosphorylation event. Finally, we found that protection against mitochondrial depolarization is independent of Hsp27 phosphorylation. Collectively, these results suggest an intimate association among p38, PKC δ , and Hsp27 and uncover a novel bimodal phosphorylation pattern in Hsp27, further elucidating the mechanism by which apigenin regulates cell death.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and cell culture

All cell lines were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ in media supplemented with 100 µg/mL penicillin, and 100 µg/mL streptomycin (P/S; BioWhittaker, Walkersville, MD). Monocytic leukemia THP-1 cells were maintained in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT). For all experiments, cells were seeded at a concentration of 0.5×10^6 cells/mL 24 h prior to treatment. Apigenin, the diluent dimethyl sulfoxide (DMSO), the phosphatase inhibitors: Na-glycerophosphate, Na-pyrophosphate, NaF, orthovanadate; dithiothreitol (DTT), and the protease inhibitors: phenylmethyl sulfonyl fluoride (PMSF), chymostatin, pepstatin, leupeptin, antipain were obtained from Sigma-Aldrich (St. Louis, MO). The substrate of caspase-3, DEVD-AFC (AFC138), and the caspase-3 inhibitor, DEVD-FMK (FMK109), were obtained from MP Biomedicals (Solon, OH). The inhibitors of p38, SB203580 (559389), MEK-1, PD98059 (513000), and the PKC δ inhibitor, rottlerin (557370), were obtained from Calbiochem (San Diego, CA).

2.2 Immunoblot and coomassie staining

Whole cell lysates were prepared by vortexing in Buffer B (50 mM HEPES pH 7.4, 2.5 mM EGTA, 1 mM EDTA, 150 mM NaCl, 10% Glycerol, 0.1% Tween 20 containing 50 mM NaF, 10 mM Na glycerophosphate, 5 mM Na pyrophosphate, 1 mM orthophosphate, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml of protease inhibitors: chymostatin,

pepstatin, leupeptin, antipain and phosphatase inhibitors) for 1 h at 4 °C. Lysates were then centrifuged at 13,200 x g for 10 min at 4 °C. Supernatants were removed from pellets and stored at -80 °C until further analysis. Polypeptides from equal amounts of protein, as determined by the Bradford method, were separated by SDS-PAGE. Gels were stained over night with Coomassie stain (0.025% (w/v) Coomassie G-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid) followed by destaining solution (30% (v/v) methanol, 10% (v/v) glacial acetic acid). For immunoblot analysis, polypeptides were transferred onto nitrocellulose membranes (25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol) and probed with antibodies of interest followed by horseradish peroxidase conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Immunoblot quantitation was performed with Biorad Quantity One software (Hercules, CA). Phospho-ERK (9101), total ERK (9102), phospho-p38 (9211), total p38 (9212), phospho-Hsp27 (Ser78) (2405), phospho-Hsp27 (Ser82) (2401), and total Hsp27 (2442) antibodies were obtained from Cell Signaling (Boston, MA). PKC δ antibody (SC-937) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β -Tubulin (05-661) was obtained from Upstate (Charlottesville, VA).

2.3 Caspase-3 activity assay

Lysates from 3×10^6 cells were prepared and incubated in a cytotbuffer as previously described (27). The activity of caspase-3 in a 10 μ g protein sample was determined using the DEVD-AFC substrate. Released AFC were measured using a

Cytofluor 400 fluorimeter (Filters: excitation 400 nm, emission 508 nm; Perspective Co., Framingham, MA).

2.4 Immunoprecipitation and *in vitro* kinase assay and phosphorylation of recombinant Hsp27

Following treatment with apigenin or DMSO, lysates were prepared as described for immunoblot analysis. Immunoprecipitation with anti-PKC δ antibodies were carried out as previously described (30). After immunoprecipitation, kinase assays were performed by incubating protein A-loaded beads for 1 h at 37 °C in the presence of 20 μ L kinase assay buffer (25 mM Hepes pH 7.3, 10 mM MnCl₂, 1 mM MgCl₂, 1 mM DTT) containing 5 μ Ci of $\{\gamma^{32}\text{P}\}$ ATP (Perkin Elmer, Boston, MA), 0.5 mM ATP. To each reaction, 5 μ g of histone H2B (Boehringer Mannheim, Roche, Indianapolis, IN) was added as exogenous substrate. Reactions were stopped by the addition of 10 mL of 5X Laemmli buffer. Samples were boiled for 5 min and loaded onto SDS-PAGE.

For phosphorylation of recombinant Hsp27, recombinant PKC δ or heat-inactivated recombinant PKC δ was incubated with recombinant wild-type Hsp27 for 1 h at 30 °C in the presence of 10 μ L of kinase assay buffer containing 10 μ Ci of $\{\gamma^{32}\text{P}\}$ ATP.

2.5 Bacterial cell culture

Bacterial cells containing selected clones were cultured overnight at 37°C at 200 rpm in 3-5 mL of LB media containing an appropriate putative antibiotic for selection.

2.6 DNA purification and restriction enzyme digestion

Plasmid DNA was purified (45) for screening and transfections using QIAprep Miniprep Kit (QIAGEN, Valencia, CA) and eluted in 50 μ L dH₂O. Plasmids were digested with combinations of restriction enzymes (NEB, Beverly, MA) in bovine serum albumin (NEB, Beverly, MA), enzyme reaction buffer, (NEB, Beverly, MA), and dH₂O for 2 hrs at 37 °C.

2.7 Fragment isolation

DNA was run on a 1% (w/v) agarose gel (Roche, Basel, Switzerland) containing ethidium bromide (0.5 μ g/ μ L) (Sigma Aldrich Corp., St. Louis, MO) to stain the DNA. DNA was loaded with a 1 kb plus marker (Invitrogen, Carlsbad, CA) and electrophoresed for 40 min at 80 volts. Fragments were purified from the agarose using a DNA gel extraction kit (QIAGEN).

2.8 DNA ligation

Vector and insert DNA were ligated to create a desired chimeric plasmid. Purified DNA insert fragments and vector were combined in a 2 to 1 ratio with T4 DNA ligase (1 U/ μ L) (Roche, Basel, Switzerland), T4 ligase reaction buffer [50 mM Tris-HCL, (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 20 μ g/ml BSA] (Roche, Basel, Switzerland) and dH₂O in a 10 μ L volume and incubated overnight at 16 °C.

2.9 Transformation of *E. coli*

Amplification of clones was carried out by transforming into 50 μ L *E. coli* Top10F competent cells (Invitrogen). Transformations were performed by combining ligation mixture (DNA, T4 ligase, ligation buffer, H₂O) and cloning cells, *E. coli* Top10F for 30 min on ice. Heat shock was performed at 42 °C for 1 min followed by incubation on ice for 2 min. One mL S.O.C. media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) (Invitrogen) was added to mixture, and mixture was incubated at 37 °C and 200 rpm for 1 h. Cells were plated on LB-agar plates containing antibiotics specific to vector and grown overnight at 37 °C. Colonies were screened by restriction analysis of miniprep DNA.

2.10 Hsp27 purification under denaturing conditions

Bacteria containing 6xHis-tagged full-length Hsp27 3A (S15A, S78A, S82A) (pQE32-Hsp27 3A), or Hsp27 3D (S15D, S78D, S82D) (pQE32-Hsp27 3D) were grown in TB media with appropriate antibiotics and 2% (w/v) glucose to an OD of approximately A₅₅₀ 0.5. Protein expression was induced with 1mM IPTG for 3 h at 37 °C. Cells were collected and washed once in complete sonication buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 5 mM β -mercaptoethanol, 1% Tween 20, and protease inhibitors (2 μ g/mL chymostatin, pepstatin, leupeptin, antipain), and 1 mM PMSF). Cells were resuspended in buffer B (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 1 μ L/mL RNase and DNase, pH 8.0) and incubated for 30 min at rt. Cells were then sonicated until lysed. Lysates were allowed to bind to Ni²⁺-NTA beads (QIAGEN) in the presence of RNase and DNase (1 μ L/mL) for 90 min at rt. After binding, the lysate-resin mixture was

loaded on an empty column and allowed to settle. The flow through was collected and the resin was washed twice with buffer C (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 1 µL/mL RNase and DNase, pH 6.3). Elutions were collected dropwise with buffer D (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 1 µL/mL RNase and DNase, pH 5.9) and buffer E (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 1 µL/mL RNase and DNase, pH 4.5). Elution fractions containing recombinant Hsp27 were identified by SDS-PAGE. The purified proteins underwent two series of dialysis. The buffer for the first dialysis consisted of 25 mM NaH₂PO₄, 100 mM NaCl, 1% (v/v) Glycerol, 0.1 mM PMSF, pH 8.5. Dialysis was performed for 20 h at 4 °C, changing to fresh buffer after 3 h and again after 15 h. The buffer for the second dialysis consisted of 20 mM Tris pH 7.4, 10 mM NaCl, 1mM EDTA, 1 mM DTT. Dialysis was performed for 4 h at 4 °C, changing the buffer three times during that time. Proteins were quantitated using a BSA standard and identified by immunoblot analysis.

2.11 Transient Transfection

Transfection of plasmids into THP-1 monocytic cells was performed as previously described (64). THP-1 cells were washed in PBS and resuspended in the specified electroporation buffer (Amaza, Cologne, Germany) to a final concentration of 5 x 10⁶ cells/mL. One µg of empty vector (pCDNA3-5myc) and vector containing wild type full-length Hsp27 (pCDNA3-Hsp27-wt-FL-5myc), full-length Hsp27 3A (S15A, S78A, S82A) (pCDNA3-Hsp27-3A-FL-5myc), or Hsp27 3D (S15D, S78D, S82D) (pCDNA3-Hsp27-3D-FL-5myc) was mixed with 0.1 mL of cell suspension and nucleofected using the Amaza Nucleofector™ according to manufacturer's specification.

After transfection, the cells were immediately transferred into 3 mL of RPMI-medium with 10% (v/v) FBS and 1% (w/v) penicillin and streptomycin.

2.12 Flow Cytometry

Twenty-four hours after transfection, the cells were treated with 50 μ M apigenin for 1 h to induce mitochondrial membrane depolarization. For detection of mitochondrial depolarization, a cell suspension was adjusted to a density of 1×10^5 cells/mL and incubated in annexin V binding buffer (BD Biosciences, Franklin Lakes, NJ) with 1% FBS for 15 min at room temperature in the dark with 5 μ g/ μ L of the mitochondrial membrane potential specific fluorescent probe JC-1. Flow cytometry analysis was performed using BD Biosciences FACSCalibur using Cellquest version 3.3 software.

2.13 Statistical Analysis

All data are expressed as mean \pm SEM and Student's t-test comparisons were conducted to analyzed statistical significance. Statistical significance is stated in the text.

CHAPTER 3

REGULATION OF HSP27 PHOSPHORYLATION DURING APIGENIN-INDUCED APOPTOSIS

Phosphorylation is known to be a critical and ubiquitous post-translational protein modification. Essential in signal transduction, phosphorylation has also been implicated in regulation of various proteins through the induction of conformational changes. Of particular interest, the phosphorylation of Hsp27 is thought to induce the dissociation of large Hsp27 oligomers to smaller multimers and dimers (73) and affect the affinity of the protein for its substrates (74). Therefore, phosphorylation, and the regulation of this phosphorylation, may play a critical role in Hsp27's anti-apoptotic activity. In this aim, we seek to determine the molecules responsible for regulating Hsp27 phosphorylation during apigenin-induced apoptosis.

3.1 Hsp27 exhibits a bimodal phosphorylation profile during apigenin-induced apoptosis.

It is known that Hsp27 becomes phosphorylated within minutes of exposure to stress (75) at three different serine residues, Ser-15, Ser-78, and Ser-82 with Ser-78 and Ser-82 being the most physiologically relevant phosphorylation sites (67, 68). In order to study the effect of apigenin on the phosphorylation status of Hsp27, we first determined the expression profile of phosphorylated Ser-78 and Ser-82 isoforms of Hsp27 during apigenin-induced apoptosis. For this purpose, THP-1 cells were treated with 50 μ M apigenin for 0.25, 0.5, 1, 3, 6, and 9 h or with DMSO as diluent control for 9 h. Cell lysates were prepared and analyzed by immunoblot analysis. (Fig 3.1). We found that both phospho-Hsp27-S78 and phospho-Hsp27-S82 exhibit a bimodal expression profile during apigenin treatment. We observed that Hsp27 phosphorylation peaks at both serine residues 15 min post-apigenin treatment. Additionally, we also observed hyperphosphorylation at 6 h post-treatment following a period of diminished presence of the phosphorylated isoforms. Hsp27 is constitutively expressed and these levels remain constant throughout apigenin-induced apoptosis.

3.2 Apigenin induces the activation of p38, ERK, and PKC δ .

The involvement of the various members of the mitogen-activated protein kinase families, such as p38 and ERK, in promoting apoptosis is well characterized (76). Additionally, PKC δ is known to be a pro-apoptotic kinase in response to a variety of stimuli in a number of cell types (77). Therefore, these kinases may play yet uncharacterized roles during apigenin-induced apoptosis; in particular, through regulation of Hsp27. Phosphorylation of Hsp27 has been shown to be dependent upon p38

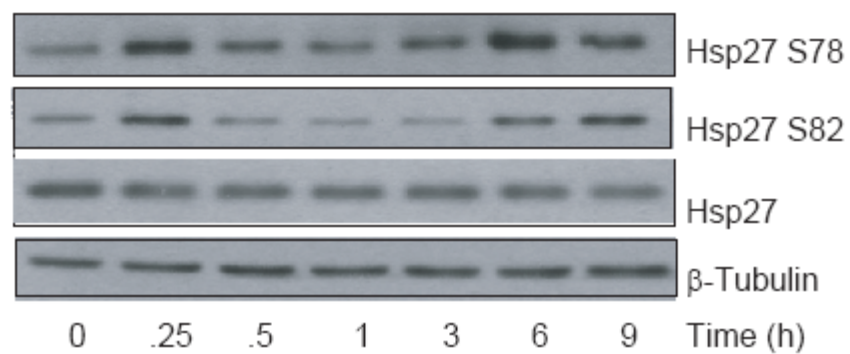


Figure 3.1 Hsp27 phosphorylation peaks at 15 min and 6 h following apigenin treatment

Lysates from THP-1 cells cultured with 50 μ M apigenin for 0.25, 0.5, 1, 3, 6, and 9 h or with DMSO as diluent control. Immunoblots with phospho-Hsp27 Ser78, phospho-Hsp27 Ser82, total Hsp27, and β -Tubulin.

activation of MK2 (67), direct interaction with PKC δ (66), and a signaling pathway involving both PKC δ and p38 (69). Moreover, ERK may play a role in Hsp27 phosphorylation as it has been identified as both a kinase directly involved in the phosphorylation of MK2 (71) and as a substrate of PKC δ (72). Therefore, the nature of the kinases involved in Hsp27 phosphorylation may be stimulus specific. To investigate a potential role for these kinases in regulation of apigenin-induced Hsp27 phosphorylation, we first determined the expression profile of phospho-p38 and phospho-ERK following apigenin treatment. THP-1 cells were treated with 50 μ M apigenin for different lengths of time or with DMSO as diluent control for 9 h. Cell lysates were prepared and analyzed by immunoblot analysis (Fig 3.2). We found that both p38 and ERK became hyperphosphorylated following apigenin treatment. As with Hsp27, we found that p38 phosphorylation exhibits a bimodal expression profile which peaks at 15 min and 6 h. In contrast, we found that ERK phosphorylation increased through 6 h where it plateaus. Our results indicate a similarity between phosphorylation of p38 and Hsp27 at both 15 min and 6 h post-apigenin treatment and between ERK and Hsp27 at 6 h post-treatment. Together these findings suggest potential roles for p38 and ERK in Hsp27 phosphorylation.

We next sought to determine the kinase activity of PKC δ during apigenin-induced apoptosis. THP-1 cells were treated with apigenin under similar conditions as above and cell lysates were prepared and analyzed by *in vitro* kinase assay to determine PKC δ 's ability to phosphorylate its substrate histone H2B (Fig 3.3). We found that PKC δ has maximal kinase activity 6 h post-apigenin treatment. In contrast, PKC δ has little kinase

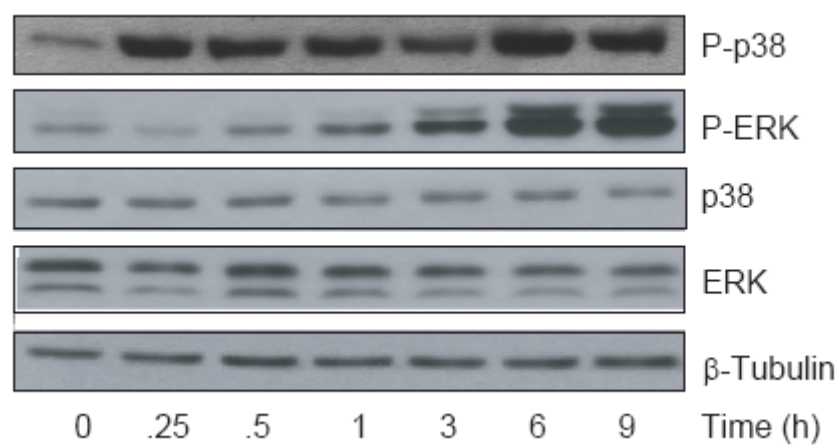


Figure 3.2 p38 MAPK and ERK phosphorylation during apigenin-induced apoptosis

Lysates from THP-1 cells cultured with 50 μ M apigenin for 0.25, .5, 1, 3, 6, and 9 h or with DMSO as diluent control. Immunoblots with phospho-p38, phospho-ERK, total p38, total ERK, and β -Tubulin.

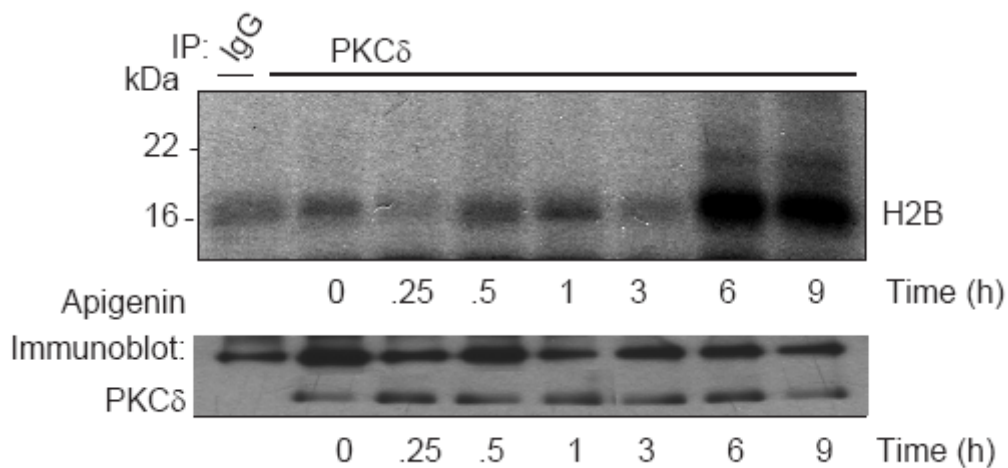


Figure 3.3 Kinase activity of PKC δ during apigenin-induced apoptosis

Lysates from THP-1 cells cultured with 50 μ M apigenin for 0.25, 0.5, 1, 3, 6, and 9 h or with DMSO as diluent control. Cells were immunoprecipitated (IP) with anti-PKC δ antibodies or an isotype control (IgG) and subjected to *in vitro* kinase assays in the presence of histone H2B and [γ^{32} P]ATP. Phospholabeled proteins were visualized by autoradiography. Immunoblots with PKC δ served as IP-loading control.

activity prior to this time point. This result suggests a potential role for PKC δ in Hsp27 phosphorylation following 6 h apigenin treatment.

3.3 Hsp27 phosphorylation is p38-dependent following 15 min apigenin treatment.

Phosphorylation profiles of Hsp27 and p38 following apigenin treatment (Fig 3.1, Fig 3.2, respectively) indicate a potential role for p38 in the phosphorylation of Hsp27 at both 15 min and 6 h post-treatment. We first sought to determine the role of p38 in Hsp27 phosphorylation following a 15 min apigenin treatment. For this purpose, we aimed to determine the effects of pharmacological inhibition of p38 on Hsp27 phosphorylation 15 min post-apigenin treatment. THP-1 cells were pretreated with 10 μ M of the p38 inhibitor SB203580 or with DMSO as diluent control for 1 h. Following pretreatment, cells were treated with 50 μ M apigenin for 15 min. Cell lysates were prepared and analyzed by immunoblot analysis (Fig 3.4). We found that pretreatment with SB203580 prior to a 15 min apigenin treatment reduced Hsp27 phosphorylation at both Ser-78 and Ser-82, as well as p38 phosphorylation (*lane 3*). These results suggest the necessity for p38 activity in Hsp27 phosphorylation following a 15 min apigenin treatment.

3.4 Hsp27 phosphorylation following 6 h apigenin treatment is characterized by residue specificity and dual pathways.

Phosphorylation profiles of Hsp27 and p38 (Fig 3.1 and Fig 3.2, respectively) and the kinase activity of PKC δ (Fig 3.3) indicate potential roles for p38 and PKC δ in the phosphorylation of Hsp27 at 6 h post-apigenin treatment. Here we sought to determine

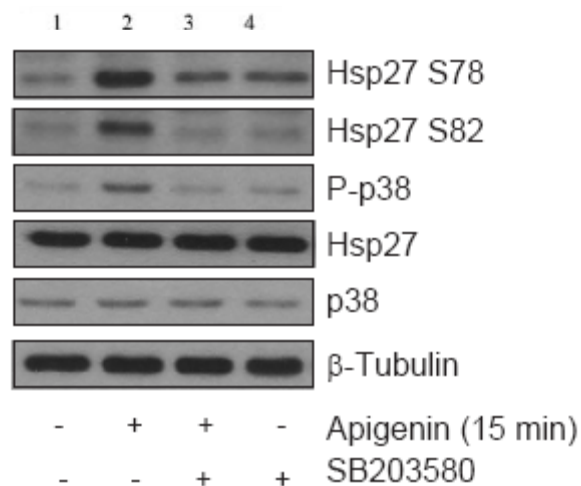


Figure 3.4 Hsp27 phosphorylation is p38 dependent following 15 min apigenin treatment

Lysates from THP-1 cells cultured with 50 μ M apigenin for 15 min following 1 h pretreatment with 10 μ M of the p38 pharmacological inhibitor SB203580 (*lane 3*). Immunoblots with phospho-Hsp27 Ser78, phospho-Hsp27 Ser82, phospho-p38, total Hsp27, total p38 and β -Tubulin.

the role of p38 and PKC δ in Hsp27 phosphorylation following 6 h apigenin treatment. For this purpose, we first sought to determine the effects of pharmacological inhibition of p38 and PKC δ on Hsp27 phosphorylation 6 h post-apigenin treatment. THP-1 cells were pretreated with either 10 μ M of the p38 inhibitor SB203580, 15 μ M of the PKC δ inhibitor rottlerin, both SB203580 and rottlerin, or DMSO as diluent control for 1 h. Following pretreatment, cells were treated with 50 μ M apigenin for 6 h. Cell lysates were prepared and analyzed by immunoblot analysis. We found that pretreatment with SB203580 prior to 6 h apigenin treatment significantly reduced Hsp27 phosphorylation at both Ser-78 (Fig 3.5, *lane 3*) and Ser-82 (Fig 3.6, *lane 3*), indicating the necessity for p38 activity in Hsp27 phosphorylation following 6 h apigenin treatment. In contrast, we found that pretreatment with rottlerin under similar conditions failed to reduce Hsp27 phosphorylation at Ser-78 (Fig 3.5, *lane 4*). However, rottlerin pretreatment prior to 6 h apigenin treatment significantly reduced Hsp27 phosphorylation at the Ser-82 residue (Fig 3.6, *lane 4*), implicating a role for PKC δ in Hsp27 phosphorylation in a residue-specific manner following 6 h apigenin treatment. As expected, pretreatment with the p38 inhibitor SB203580 and the PKC δ inhibitor rottlerin significantly reduced Hsp27 phosphorylation at both serine residues (Fig 3.5, Fig 3.6, *lane 5*).

Hsp27 is known to be phosphorylated by p38 through MK2. In contrast, it has been shown that PKC δ can directly phosphorylate Hsp27 (66, 70). Our results suggest that PKC δ may directly phosphorylate Hsp27 in a residue-specific manner following 6 h apigenin treatment. To test this hypothesis, recombinant PKC δ was incubated with recombinant wild-type Hsp27 and [γ ³²P]ATP and analyzed by immunoblot analysis to

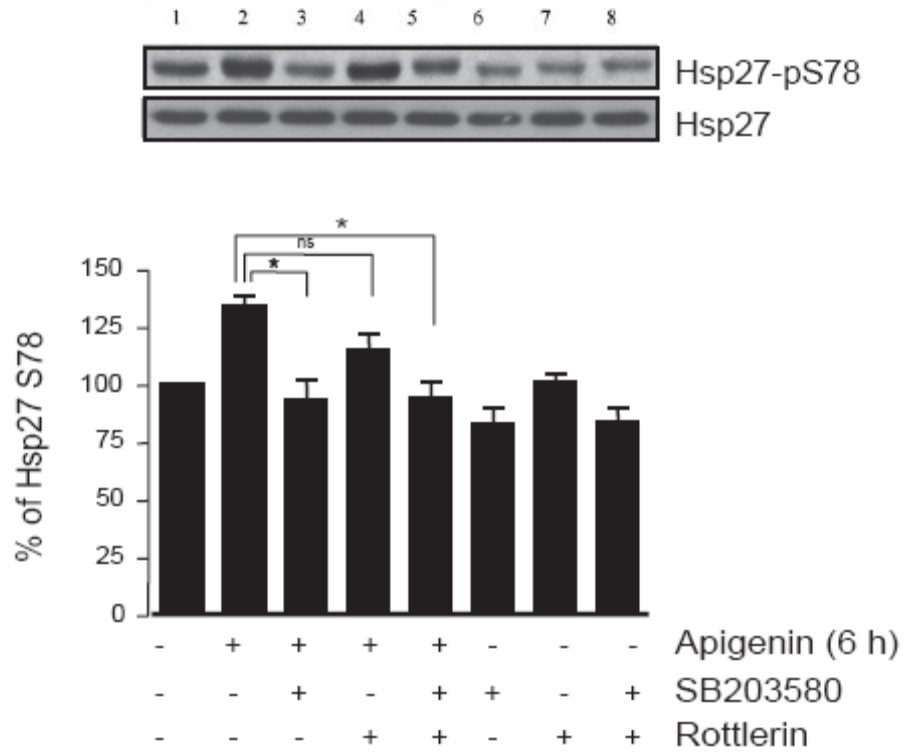


Figure 3.5 Hsp27 phosphorylation at Ser-78 is p38 dependent following 6 h apigenin treatment

Lysates from THP-1 cells cultured with 50 μ M apigenin for 6 h following 1 h pretreatment with 10 μ M of the p38 pharmacological inhibitor SB203580 (*lane 3*), the PKC δ inhibitor rottlerin (*lane 4*), or both (*lane 5*). Immunoblots with phospho-Hsp27 Ser-78 and total Hsp27. Densitometry data normalized by the loading control is represented as Mean \pm SEM (N=6, $\star p \leq 0.01$, NS denotes non-statistical significance)

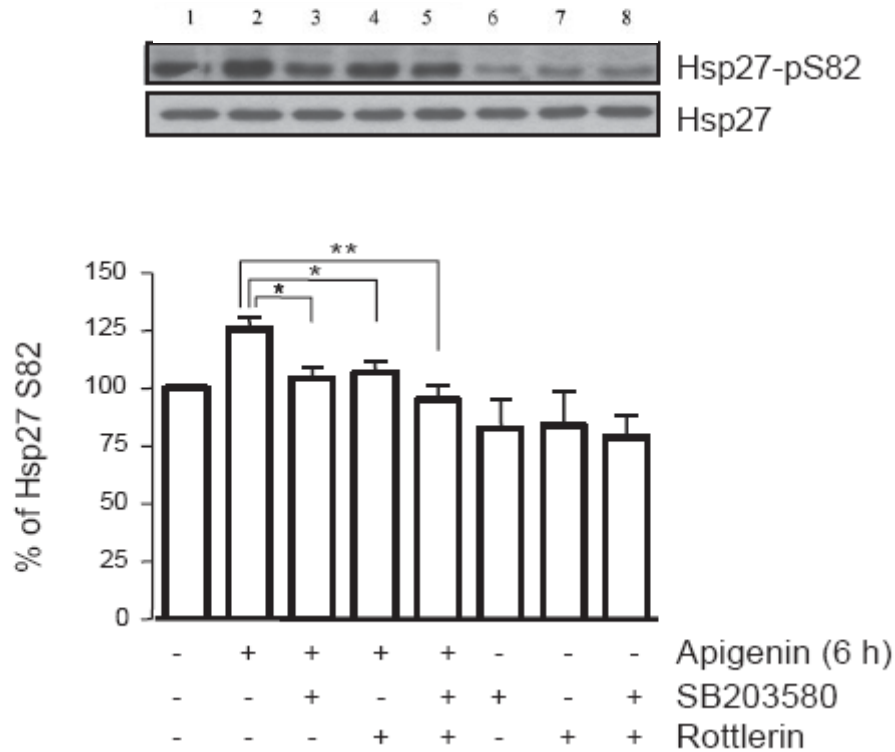


Figure 3.6 Hsp27 phosphorylation at Ser-82 is p38 and PKC δ dependent following 6 h apigenin treatment

Lysates from THP-1 cells cultured with 50 μ M apigenin for 6 h following 1 h pretreatment with 10 μ M of the p38 pharmacological inhibitor SB203580 (*lane 3*), the PKC δ inhibitor rottlerin (*lane 4*), or both (*lane 5*). Immunoblots with phospho-Hsp27 Ser82 and total Hsp27. Densitometry data normalized by the loading control is represented as Mean \pm SEM (N=6, * $p \leq 0.01$ and ** $p \leq 0.05$)

determine PKC δ 's ability to phosphorylate Hsp27 (Fig 3.7). As expected, we found that PKC δ could not phosphorylate Hsp27 at Ser-78. In contrast, we found that PKC δ can directly phosphorylate Hsp27 at Ser-82. Together these findings suggest that PKC δ can phosphorylate Hsp27 selectively at Ser-82 *in vitro*.

PKC δ has also been shown to act upstream of p38 activation (69). Therefore, we next sought to determine the relationship between PKC δ and p38 in apigenin-induced Hsp27 phosphorylation. For this purpose, THP-1 cells were treated with pharmacological inhibitors of p38 and PKC δ as indicated above followed by 6 h treatment with apigenin. Cell lysates were prepared and first analyzed by immunoblot analysis (Fig 3.8A). We found that while the p38 inhibitor SB203580 expectantly reduces p38 phosphorylation (lane 3), the PKC δ inhibitor rottlerin fails to reduce p38 phosphorylation (lane 4), indicating that PKC δ is not an upstream regulator of p38 activation following 6 h apigenin treatment. We also analyzed the same samples by *in vitro* kinase assay to determine PKC δ 's kinase activity following pretreatment with p38 and PKC δ pharmacological inhibitors (Fig 3.8B). As expected, we found that pretreatment with the PKC δ inhibitor rottlerin decreased PKC δ kinase activity following 6 h apigenin treatment (lane 5). Interestingly, pretreatment with the p38 inhibitor SB203580 (lane 4) also decreased PKC δ kinase activity, suggesting that p38 may act upstream to PKC δ and may provide an additional pathway through which p38 regulates Hsp27 phosphorylation.

3.5 The effect of PKC δ on Hsp27 phosphorylation is independent of ERK activation

As stated, ERK has been identified as both a kinase directly involved in the phosphorylation of MK2 (71) and as a substrate of PKC δ (72). Additionally, our results

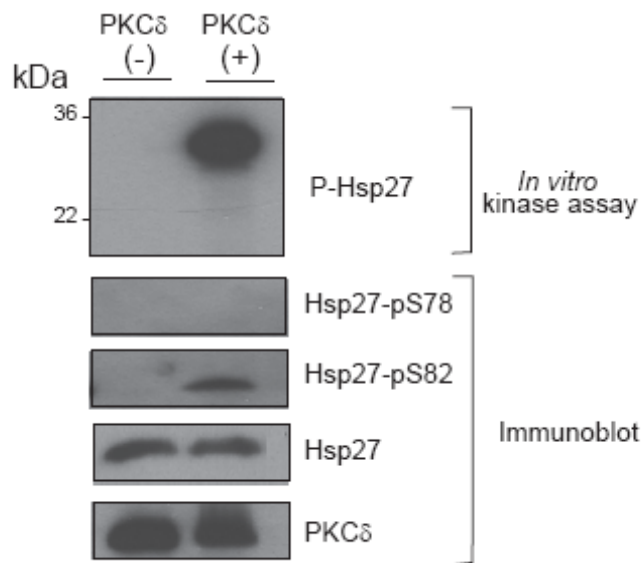


Figure 3.7 PKCδ selectively phosphorylates Hsp27 at Ser-82 *in vitro*.

Recombinant PKCδ (+) or heat-inactivated recombinant PKCδ (-) was incubated with recombinant wild-type Hsp27 and [γ^{32} P]ATP. Phospholabeled proteins were visualized by autoradiography. Immunoblots with phospho-Hsp27 Ser78, phospho-Hsp27 Ser82, total Hsp27, and PKCδ.

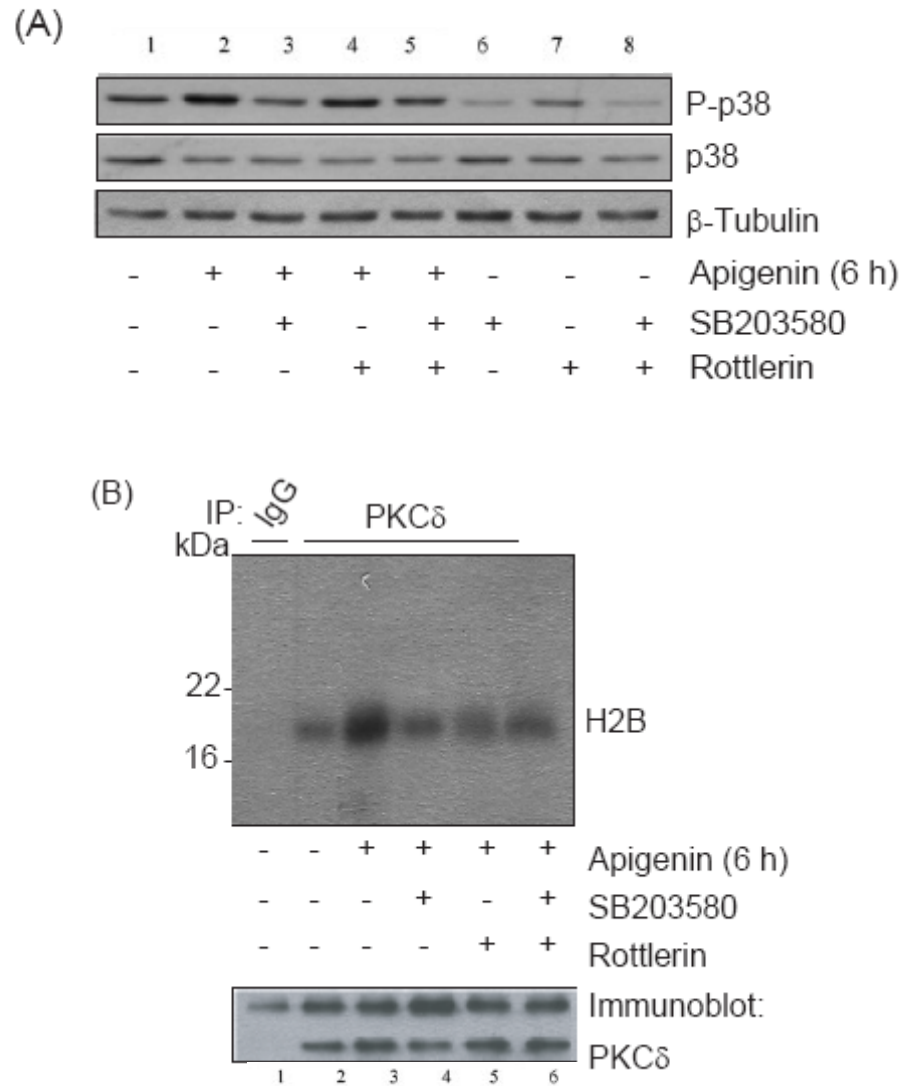


Figure 3.8 p38 acts upstream of PKC δ following 6 h apigenin treatment.

Lysates from THP-1 cells cultured with 50 μ M apigenin for 6 h following 1 h pretreatment with 10 μ M of the p38 pharmacological inhibitor SB203580 (*lane 3 [A], lane 4 [B]*), the PKC δ inhibitor rottlerin (*lane 4 [A], lane 5 [B]*), or both (*lane 5 [A], lane 6 [B]*). (A) Immunoblots with phospho-p38, p38 total, and β -Tubulin. (B) *In vitro* kinase assays of IP-PKC δ in the presence of H2B and [γ^{32} P]ATP. Immunoblots with PKC δ served as loading control.

show that ERK phosphorylation is greatest at 6 h following apigenin treatment, indicating a potential role for ERK in apigenin-induced Hsp27 phosphorylation at 6 h. In order to assess the role of ERK in Hsp27 phosphorylation we first sought to determine if ERK was a downstream target of PKC δ . For this purpose, we sought to determine the effects of pharmacological inhibition of PKC δ on ERK phosphorylation 6 h post-apigenin treatment. THP-1 cells were pretreated with 15 μ M of the PKC δ inhibitor rottlerin or with DMSO as diluent control for 1 h. Following pretreatment, cells were treated with 50 μ M apigenin for 6 h. Cell lysates were prepared and analyzed by immunoblot analysis (Fig 3.9A). We found that inhibition of PKC δ with rottlerin reduced ERK phosphorylation following 6 h apigenin treatment (*lane 3*), identifying ERK as a potential downstream effector of PKC δ during apigenin-induced apoptosis.

To further assess the relationship between PKC δ and ERK we sought to determine the effects of pharmacological inhibition of ERK on Hsp27 phosphorylation. THP-1 cells were pretreated with 25 μ M of the ERK inhibitor PD98059 or with DMSO as diluent control for 1 h. Following pretreatment, cells were treated with 50 μ M apigenin for 6 h. Cell lysates were prepared and analyzed by immunoblot analysis (Fig 3.9B). We found that inhibition of ERK did not reduce Hsp27 phosphorylation at Ser-78 and Ser-82 (*lane 3*). These findings suggest that a role for PKC δ in Hsp27 phosphorylation is independent of ERK, further providing evidence that PKC δ may directly phosphorylate Hsp27 during apigenin-induced apoptosis.

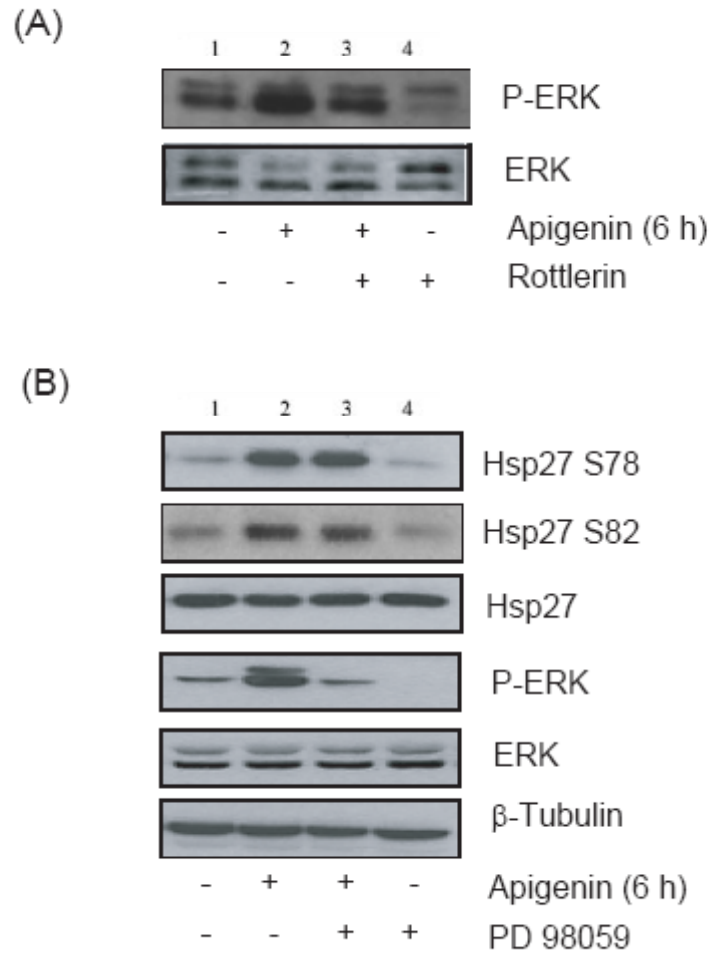


Figure 3.9 PKC δ effect on Hsp27 phosphorylation is independent of the downstream target ERK

Lysates from THP-1 cells cultured with 50 μ M apigenin for 6 h following 1 h pretreatment with (A) 15 μ M of the PKC δ inhibitor rottlerin (*lane 3*) or (B) 25 μ M of the ERK pharmacological inhibitor PD98059 (*lane 3*). Immunoblots with phospho-Hsp27 Ser-78, phospho-Hsp27 Ser-82, phospho-ERK, total Hsp27, total ERK and β -Tubulin.

3.6 Generation of the PKC δ constitutively active catalytic fragment is not necessary for Hsp27 phosphorylation.

PKC δ has been shown to phosphorylate and activate caspase-3 (30). PKC δ has also been identified as a substrate of caspase-3 and its proteolytic cleavage results in a constitutively active catalytic domain necessary for apoptosis (31, 32). Therefore, caspase-3 may play a role in Hsp27 phosphorylation through PKC δ proteolytic activation. We first sought to determine the effect of inhibition of caspase-3 activity on PKC δ kinase activity. THP-1 cells were pretreated with 25 μ M of the caspase-3 inhibitory peptide DEVD-FMK or with DMSO as diluent control for 1 h. Following pretreatment, cells were treated with 50 μ M apigenin for 6 h. Cell lysates were prepared and analyzed by an *in vitro* kinase assay to determine PKC δ 's kinase activity following pretreatment with the caspase-3 inhibitor (Fig 3.10A). We found that inhibition of caspase-3 activity decreases PKC δ kinase activity (*lane 4*), suggesting that PKC δ does not get cleaved to its constitutively active catalytic fragment in the presence of the caspase-3 inhibitory peptide. To further assess the role of caspase-3, we determined the effects of inhibition of caspase-3 activation on Hsp27 phosphorylation. THP-1 cells were treated with the inhibitory peptide of caspase-3 as indicated above followed by 6 h treatment with apigenin. Cell lysates were prepared and analyzed by immunoblot analysis (Fig 3.10B). We found that inhibition of caspase-3 activity failed to reduce Hsp27 phosphorylation at both serine residues, as well as p38 phosphorylation (*lane 3*), indicating that caspase-3 activity is not necessary for Hsp27 phosphorylation. DEVD-AFC analysis of these samples confirms the efficiency of caspase-3 inhibition

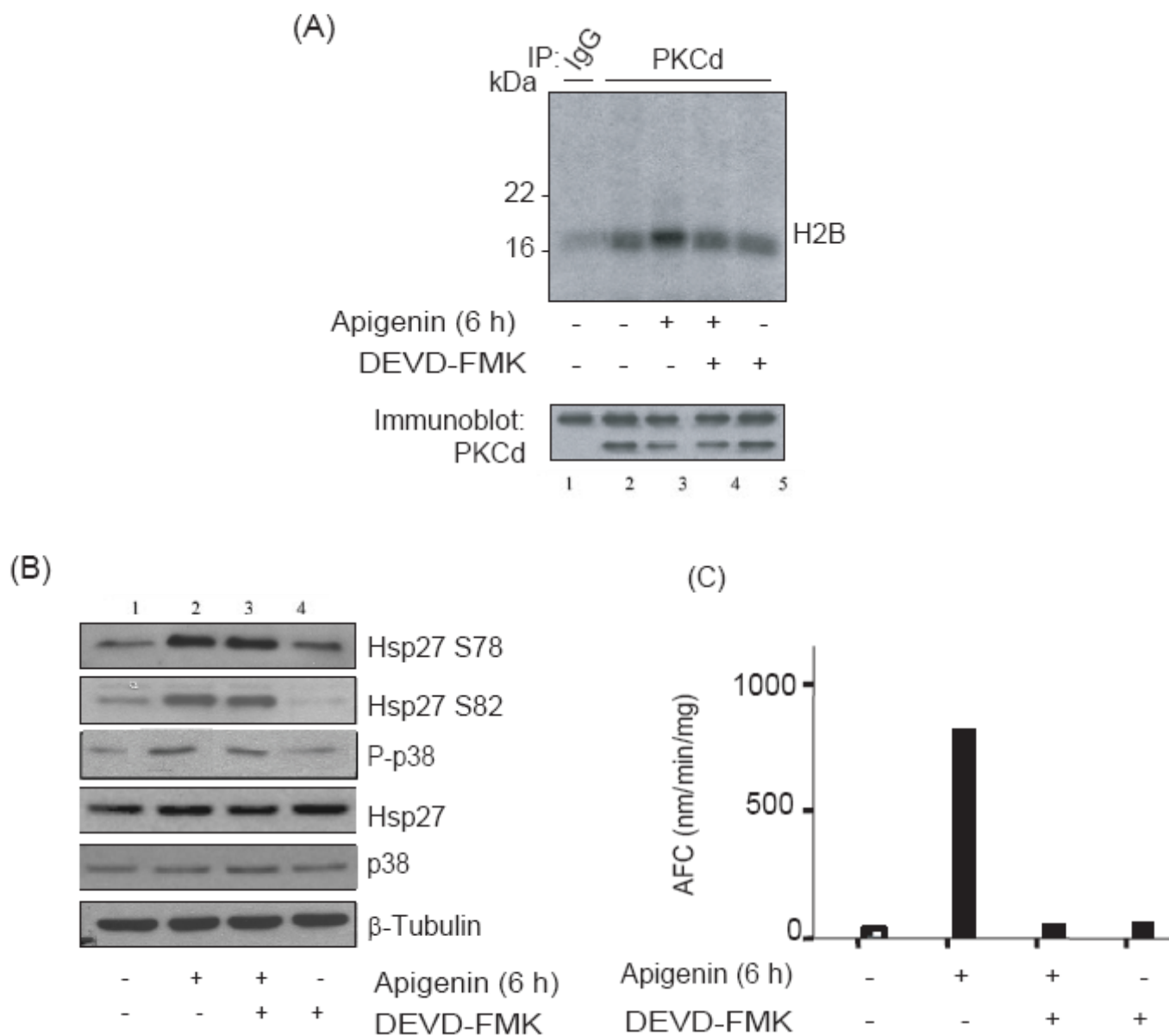


Figure 3.10 PKC δ activity, but not Hsp27 and p38 phosphorylation, is dependent on caspase-3 activity following 6 h apigenin treatment

Lysates from THP-1 cells cultured with 50 μ M apigenin for 6 h following 1 hr pretreatment with 25 μ M of the caspase-3 inhibitory peptide DEVD-FMK (lane 3 [A, B], lane 4 [C]). (A) *In vitro* kinase assays of IP-PKC δ in the presence of H2B and [γ ³²P]ATP. (B) Immunoblots with phospho-Hsp27 Ser78, phospho-Hsp27 Ser82, phospho-p38, total Hsp27, total p38 and β -Tubulin. (C) DEVD-AFC analysis of caspase-3 activity.

(Fig 3.10C). Together these results suggest that the non-constitutively active full-length PKC δ may phosphorylate Hsp27 at Ser-82 following apigenin treatment.

CHAPTER 4

ROLE OF HSP27 PHOSPHORYLATION DURING APIGENIN-INDUCED APOPTOSIS

Mitochondrial membrane depolarization occurs within minutes of exposure to apoptotic stimuli as a consequence of the mitochondrial permeability transition pore opening, preceding many other characteristic biochemical changes of apoptosis and serving as a point of no return of the apoptotic pathway (78). As a potent apoptotic stimulus, apigenin has been shown to induce mitochondrial membrane depolarization (46). Hsp27 is known to exhibit its anti-apoptotic activity at various points in the apoptotic pathway. One of the earliest anti-apoptotic functions of Hsp27 is its ability to prevent mitochondrial membrane depolarization (57). Therefore, Hsp27 may protect against apigenin-induced mitochondrial depolarization. Moreover, given that large, non-phosphorylated Hsp27 oligomers have been suggested to be required for Hsp27's anti-apoptotic function (65), phosphorylation of Hsp27 may directly affect the protein's ability to protect against mitochondrial depolarization and apoptosis. In this aim, we begin to study the role phosphorylation of Hsp27 plays during apigenin-induced phosphorylation.

4.1 Introduction of Hsp27 mutants into the 6xHis-tagged expression vector pQE32 and protein purification under denaturing conditions.

For future use to characterize Hsp27 direct interaction and association with targets, phospho-null Hsp27 3A (S15A, S78A, S82A) and phospho-mimic Hsp27 3D (S15D, S78D, S82D) recombinant proteins were prepared. For this purpose, the Hsp27 3A and Hsp27 3D clones were excised from a pENTR/D-TOPO vector and introduced into a pQE32 vector in order to add a 6xHis-tag that can be used for affinity purification (Fig 4.1). The pENTR/D-TOPO vector is used to create clones using topoisomerase to ligate a small insert with only one small sticky end. Traditionally, the vector is used for recombination into a destination vector, such as pQE32. From glycerol stocks, bacterial colonies containing pENTR/D-TOPO-Hsp27 3A (AB566) and pENTR/D-TOPO-Hsp27 3D (AB567), as well as pQE32 plasmids (AB262) were grown on antibiotic-resistant agar plates. From select colonies, plasmid DNA was isolated and digested with Bam HI and Sal I (Fig 4.2A) resulting in cut pQE32 (*lane 1*), Hsp27 3A fragment (*lane 2*, 648 bp), and Hsp27 3D fragment (*lane 3*, 648 bp). DNA extraction from the agarose gel yielded purified linearized pQE32, Hsp27 3A insert, and Hsp27 3D insert (Fig 4.2B). Following DNA extraction, linearized pQE32 was ligated to Hsp27 3A and Hsp27 3D inserts in the presence of T4 ligase. The ligation was used to transform *E. coli* Top10F competent cells, and colonies were selected on LB-agar plates containing ampicillin. DNA was purified and screened by sequencing and restriction enzyme analysis with Bam HI and Sal I (Fig 4.2C) with positive clones yielding inserts of 648 bp (*lane 1,2,3* [pQE32-Hsp27 3A], *lane 5,6* [pQE32-Hsp27 3D])

E. coli containing clones pQE32-Hsp27 3A and pQE32-Hsp27 3D was used for protein purification. Expression of 6xHis-tagged Hsp27 phospho-

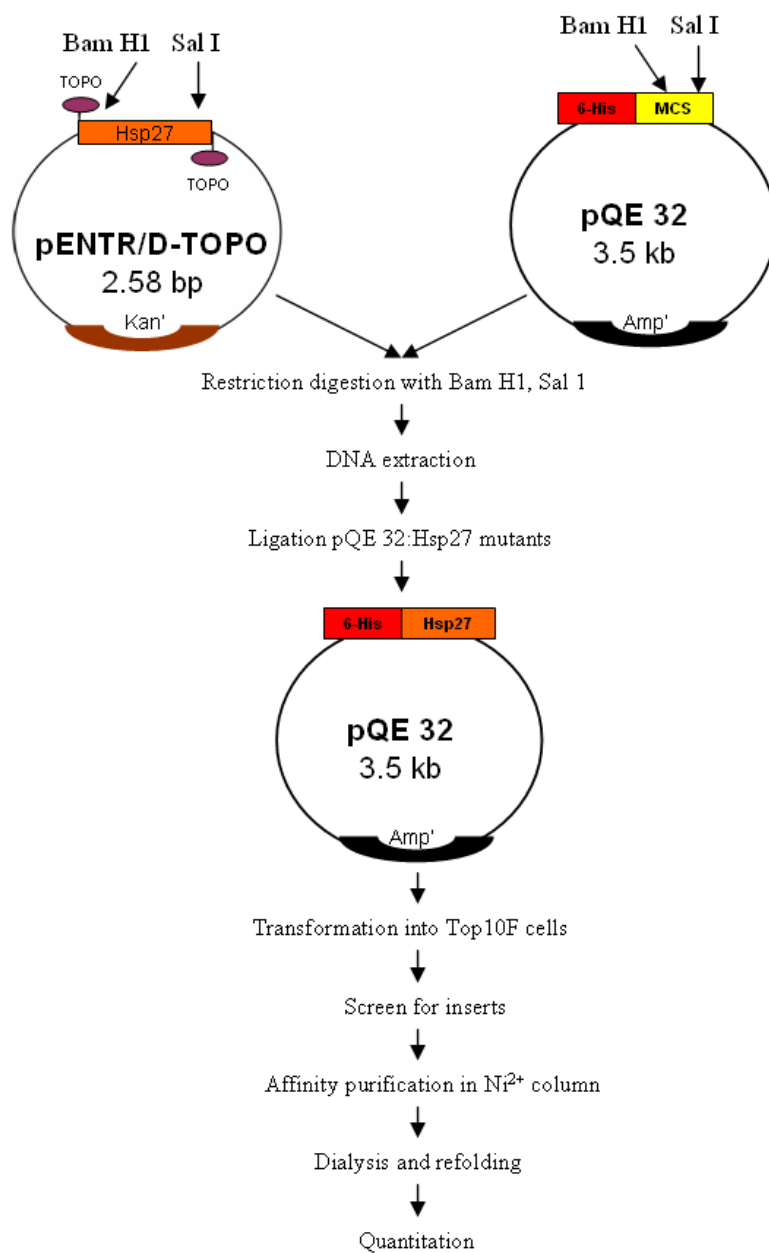


Figure 4.1 Experimental scheme.

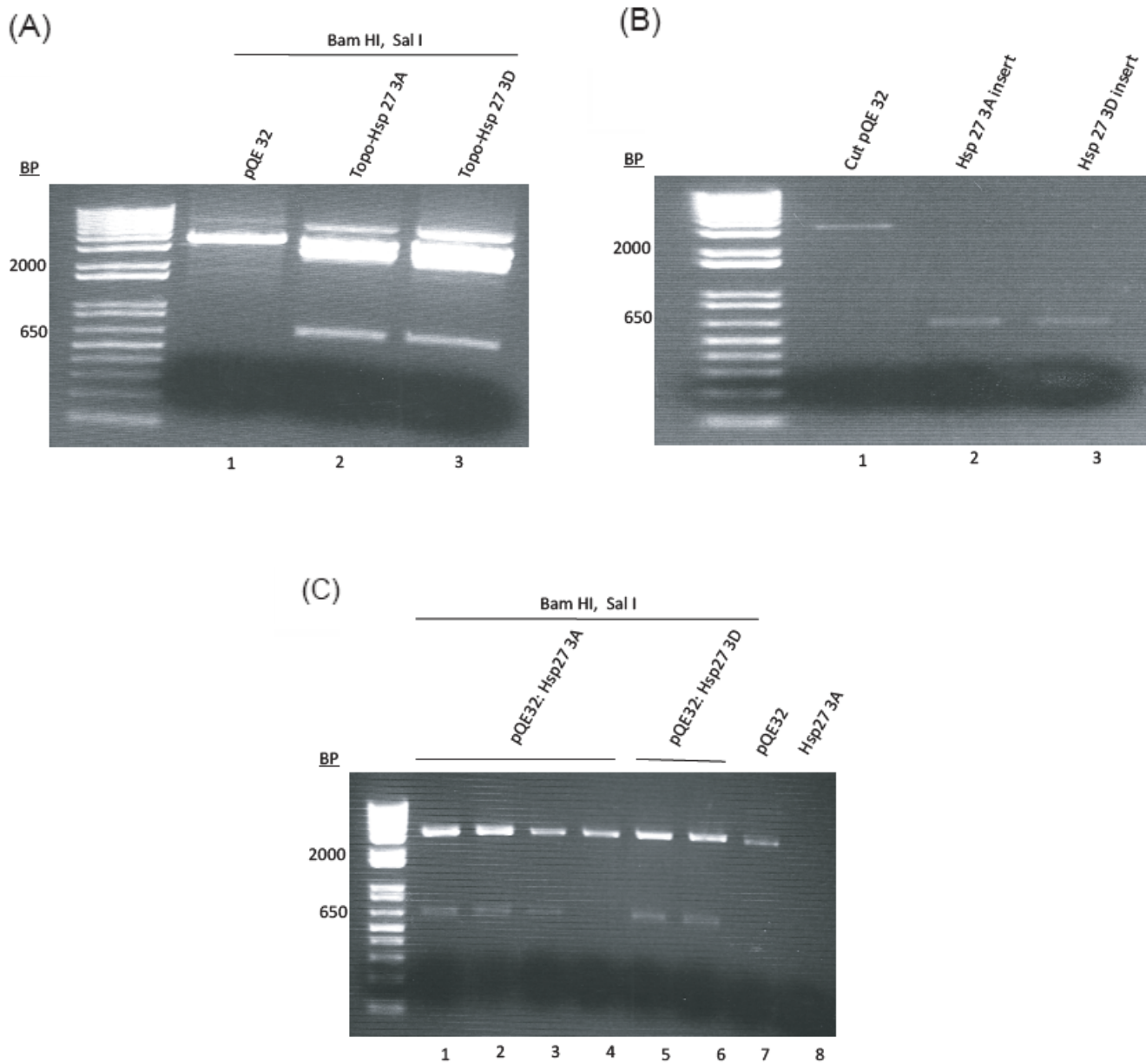


Figure 4.2 Ligation of Hsp27 3A and Hsp27 3D into the 6xHis-tagged pQE 32 vector

(A) pQE 32, Topo-Hsp27 3A, Topo-Hsp27 3D were digested with Bam HI and Sal I. (B) DNA extraction from the agarose gel yielded purified cut pQE32 and Hsp27 3A and Hsp27 3D inserts. (C) Following ligation of pQE32:Hsp27 3A and pQE32:Hsp27 3D and transformation into *E. coli* Top10F competent cells, restriction digestion with Bam HI and Sal I identified positive clones (lane 1,2,3 [pQE32-Hsp27 3A], lane 5,6 [pQE32-Hsp27 3D])

mutants was induced using IPTG and recombinant Hsp27 3A-6xHis and Hsp27 3D-6xHis was purified from bacteria using a Ni²⁺-NTA affinity column (see Material and Methods). Using increasingly acidic buffers, substantial amount of protein was obtained in the first two elutions with buffer E (Fig 4.3A *lanes 12, 13*). Following dialysis and refolding, elution fractions containing mutant Hsp27 were analyzed by SDS-PAGE and quantitated by coomassie staining against a BSA gradient (Fig 4.3B) and identified by immunoblot with total Hsp27 (Fig 4.3C).

4.2 Phosphorylation of Hsp27 is not required for Hsp27's ability to protect against apigenin-induced mitochondrial membrane depolarization.

Hsp27 is able to block apoptosis at different stages because of its interaction with different partners. The capacity of Hsp27 to interact with one or another partner seems to be determined by the oligomerization and phosphorylation status of the protein. At the earliest stages of apoptosis, Hsp27 is known to prevent mitochondrial depolarization through the suppression ROS formation by inhibiting PKC δ and upholding glutathione in its reduced form (34, 59). Therefore, phosphorylation of Hsp27 may serve to regulate Hsp27's ability to prevent mitochondrial membrane depolarization. For this purpose, we sought to determine the effect of transfection of DNA encoding wild-type Hsp27, phospho-null Hsp27 3A and phospho-mimic Hsp27 3D into THP-1 cells on membrane depolarization. Twenty-four hours after transfection of empty vector (pCDNA3-5myc), vector containing wild type full-length Hsp27 (pCDNA3-Hsp27-wt-FL-5myc), full-length Hsp27 3A (pCDNA3-Hsp27-3A-FL-5myc), or full-length Hsp27 3D (pCDNA3-Hsp27-3D-FL-5myc), cells were treated with 50 μ M apigenin for 1 h. Cells were then fixed with the mitochondrial membrane potential specific fluorescent probe JC-1 and

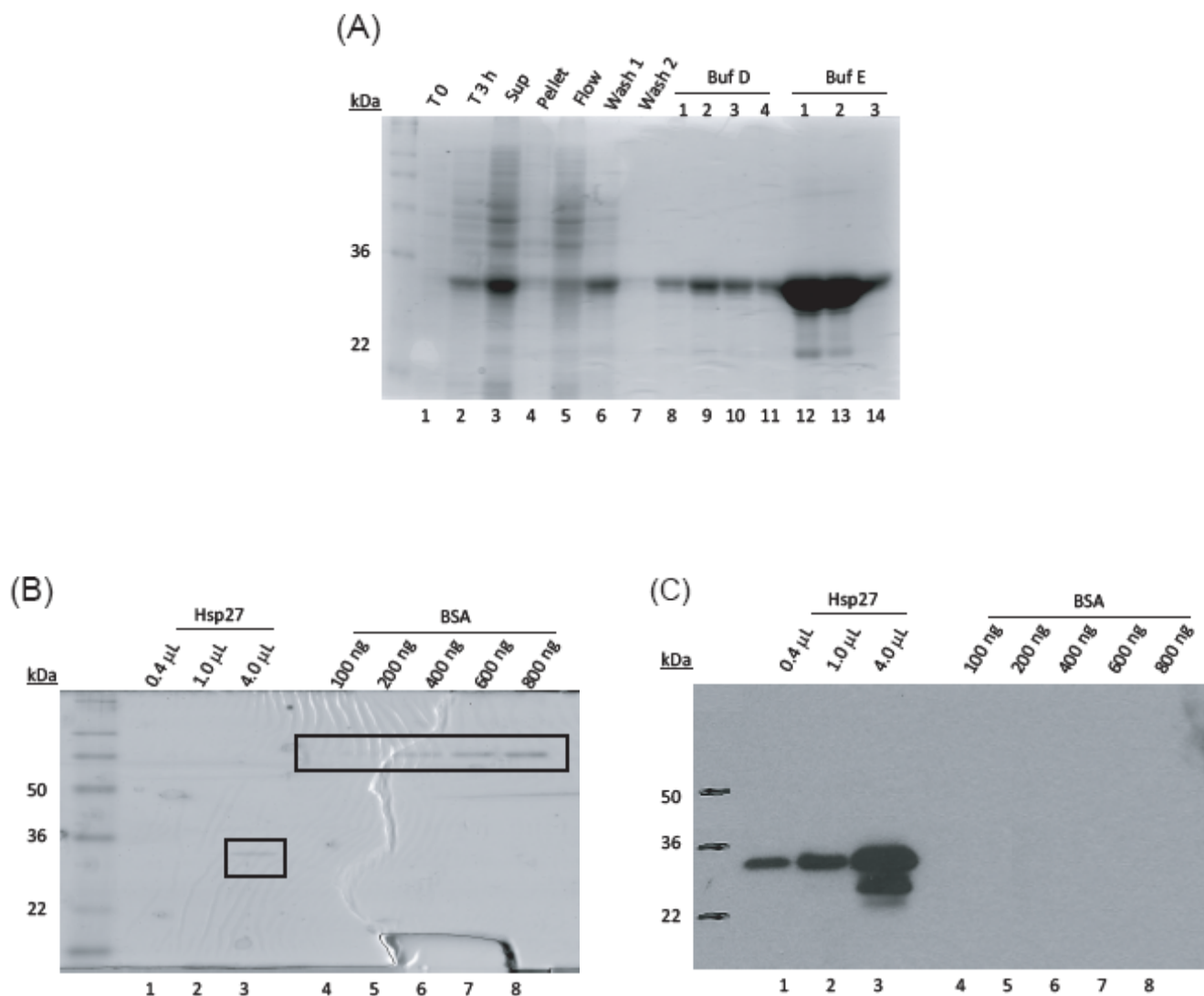


Figure 4.3 Purification of 6xHis-tagged Hsp27 under denaturing conditions

(A) Coomassie stain of the purification of Hsp27 clones using a Ni^{2+} -affinity column with elutions in a discontinuous pH gradient (buffer D pH 5.9; buffer E pH 4.5). Following refolding, elution fractions containing mutant Hsp27 were analyzed by SDS-PAGE and (B) quantitated by coomassie staining against a BSA standard and (C) immunoblotted with total Hsp27.

analyzed by flow cytometry to detect color shifts in the dye upon formation of JC-1 aggregates accompanying membrane depolarization (Fig 4.4). We found that apigenin treatment for 1 h increases mitochondrial membrane potential by 27% (Fig 4.4A). As expected, we found that introduction of Hsp27 wt-FL reduces mitochondrial depolarization to 6% (Fig 4.4B). Interestingly, we also found that both Hsp27 3A-FL and Hsp27 3D-FL (Fig 4.4C, Fig 4.4D, respectively) are able to reduce mitochondrial depolarization in the same magnitude, suggesting that phosphorylation of Hsp27 is not required for the protein's ability to protect against apigenin-induced mitochondrial depolarization.

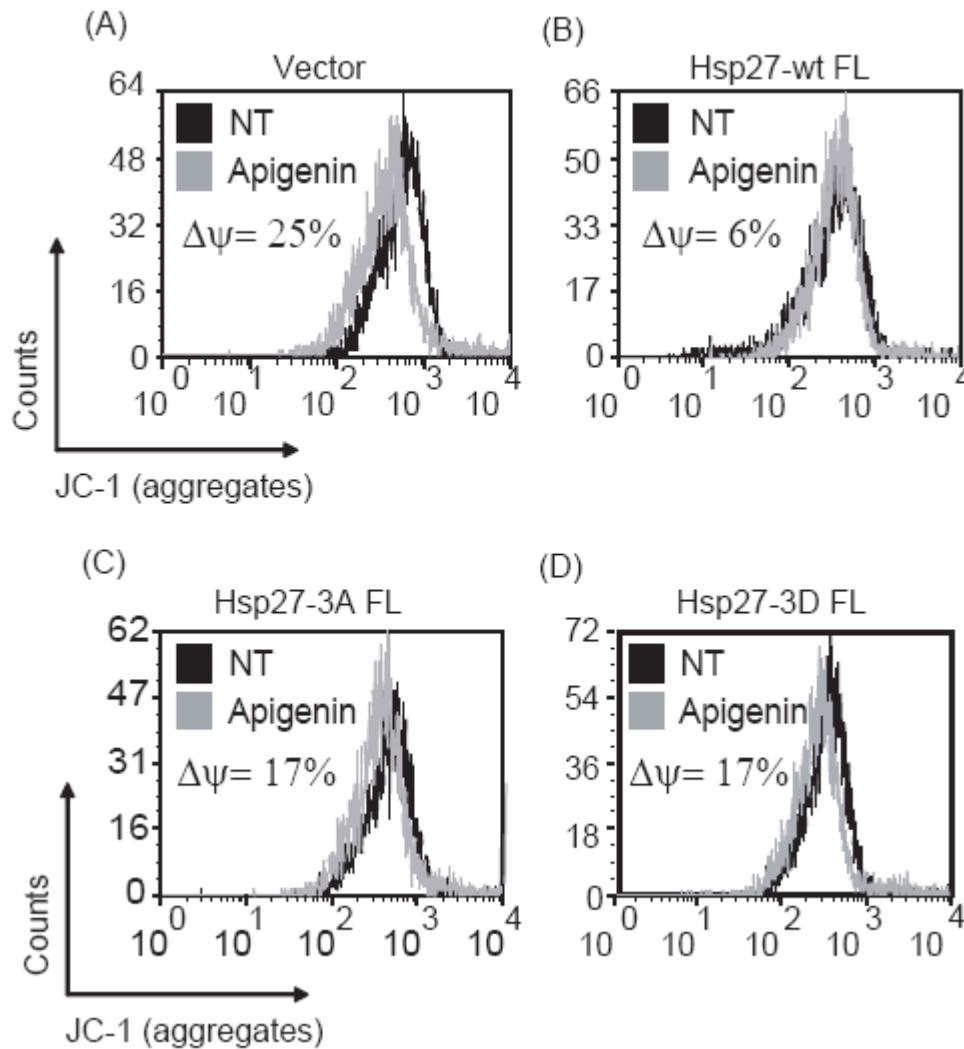


Figure 4.4 Hsp27's ability to protect against mitochondrial depolarization is independent of its phosphorylation status

THP-1 cells were transfected with (A) empty vector, (B) vector containing Hsp27-wt-FL, (C) Hsp27 3A (S15A-S78A-S82A), or (D) Hsp27 3D (S15D-S78D-S82D) and cultured with 50 μM apigenin for 1 h. Cells were fixed with JC-1 and analyzed by FACS analysis to determine changes in mitochondrial membrane potential.

CHAPTER 5

DISCUSSION

Hsp27 is a chaperone that exhibits many anti-apoptotic functions. Central to Hsp27 anti-apoptotic function is the dynamic relationship between phosphorylation, oligomerization, and association with target proteins (55, 66, 73, 74). In particular, Hsp27 is known to regulate many of the molecules involved in caspase-dependent apoptosis (60-63). During caspase-dependent apoptosis, external signals transduced through death receptors or internal signals transduced through mitochondrial dysfunction generate a signaling cascade that culminates in the proteolytic activation of effector caspases responsible for facilitating the characteristic biochemical changes observed during apoptosis (3). Among the signals more commonly responsible for apoptosis are cytotoxic drugs. Recently, our lab identified a plant flavonoid, apigenin, that has anti-cancer and anti-inflammatory properties (47). We showed that apigenin is a potent pro-apoptotic molecule in leukemia cells through localization to the mitochondria and induction of apoptosis in a PKC δ and caspase-dependent manner. In this work, we built on the model of apigenin-induced apoptosis with an emphasis on the regulation and role of Hsp27 phosphorylation.

Since many of the functions attributed to Hsp27 require its phosphorylation (74, 79, 80), the identification of the protein kinases that mediate Hsp27 phosphorylation is critical for understanding the regulation of Hsp27's multiple cellular functions. The mitogen-activated protein kinase p38 is known to be activated in response to chemotherapeutic drugs as a pro-apoptotic molecule (26) and is widely regarded as an upstream kinase of Hsp27 through activation of MK2 (81). Thus, p38 was investigated as

a potential kinase involved in apigenin-induced Hsp27 phosphorylation. Accordingly, we demonstrate that Hsp27 phosphorylation at Ser-78 and Ser-82 is p38 dependent following 15 min and 6 h apigenin treatment (Fig 3.4 – Fig 3.6). In addition, we exhibit that p38 activity following 6 h apigenin treatment is independent of caspase-3 activity (Fig 3.10B), consistent with findings that p38 activity is not essential for apigenin-induced apoptosis (47). PKC δ has also been implicated in Hsp27 phosphorylation, albeit in a stimulus-dependent manner (82). PKC δ can act as either pro-apoptotic molecule or anti-apoptotic molecule depending on the stimulus (77). Our lab has shown that PKC δ associates with and phosphorylates caspase-3, promoting apoptosis (30). Here, we identified a role for PKC δ in apigenin-induced Hsp27 phosphorylation. We demonstrate that PKC δ can directly and selectively phosphorylate Hsp27 at Ser-82 (Fig 3.7). We also find that PKC δ may act downstream of p38 in a pathway parallel to p38 activation of MK2 at 6 h post-apigenin treatment (Fig. 3.8B). In addition, we find that PKC δ exhibits its effect on Hsp27 phosphorylation independent of both downstream ERK (Fig 3.9) and cleavage by caspase-3 (Fig 3.10), suggesting that full-length PKC δ may directly phosphorylate Hsp27 at Ser-82 following 6 h apigenin treatment as a result of p38 activation.

The role played by Hsp27 as an anti-apoptotic molecule appears to be largely determined by its oligomerization and thus, phosphorylation state. It has been suggested that large non-phosphorylated oligomers are the structural organization required for Hsp27's caspase-dependent anti-apoptotic activity (65). However, phosphorylation of Hsp27 results in a shift from large, nonphosphorylated oligomers to smaller phosphorylated multimers such as dimers and this shift attenuates Hsp27's affinity for its

substrates (74). Therefore, phosphorylation may disrupt the anti-apoptotic activity of Hsp27. However, our findings suggest that Hsp27's ability to protect against apigenin-induced mitochondrial membrane depolarization (Fig 4.4) is independent of the protein's phosphorylation state. We find that the Hsp27 wt can protect against apigenin-induced mitochondrial depolarization. Moreover, phospho-null Hsp27 3A mutant and phospho-mimic Hsp27 3D mutant have similar cytoprotective abilities. Therefore; results are consistent with previous suggestions that large non-phosphorylated oligomers are required for Hsp27's anti-apoptotic activity at this early event.

According to our model of apigenin-induced apoptosis, Hsp27 is phosphorylated at both Ser-78 and Ser-82 by p38 through MK2 at 15 min and 6 h and at Ser-82 by p38 through PKC δ at 6 h. The bimodal phosphorylation of Hsp27 during apigenin-induced apoptosis is a distinct mechanism of Hsp27 regulation. The underlying significance of our findings is that different methods of regulation of Hsp27 phosphorylation could result in different fates for the phosphorylated molecule. Our findings suggest that early and late phosphorylation of Hsp27 following apigenin treatment represent distinct, and perhaps mechanistically important, roles for the phosphorylated molecule in response to apigenin-induced apoptosis. Apigenin-induced early phosphorylation may play either an anti-apoptotic or pro-apoptotic role within the cell. Upon phosphorylation by the p38 MAPK pathway, Hsp27 has been shown to protect cells from oxidative stress by upholding glutathione in its reduced form (83) as well as stabilizing actin filament dynamics following stress (80, 84). These functions may protect the cell against mitochondrial dysfunction as both oxidative stress and actin destabilization contribute to mitochondrial dysfunction and apoptosis (85, 86). However, our results indicate that

Hsp27 phosphorylation is not essential for the protein's ability to protect against mitochondrial depolarization, suggesting that early Hsp27 phosphorylation may be a stress response rather than an anti-apoptotic response. Additionally, early Hsp27 phosphorylation may play a pro-apoptotic role through its ability to cause dissociation of the molecule from its target proteins. In particular, Hsp27 phosphorylation may play a role in dissociation from its target proteins during caspase-dependent apoptosis. Our lab has shown using recombinant proteins that cleavage of the prodomain of caspase-3 occurs as early as 45 min following the addition of caspase-9 and that addition of Hsp27 attenuates this cleavage (64). Therefore, early phosphorylation of Hsp27 may play a role in promoting caspase-dependent apoptosis by alleviating Hsp27 inhibition of caspase-3. In addition, our lab has shown that Hsp27 is found mostly in the nucleus at 8 h during spontaneous monocyte apoptosis. This may suggest a role for the late Hsp27 phosphorylation as phosphorylation of Hsp27 has been shown to affect the protein's ability to translocate to the nucleus (79, 87). Additionally, the ability to selectively phosphorylate Hsp27 at either Ser-78 or Ser-82 following 6 h apigenin treatment could result in drastically different cellular fates. Indeed, specific phosphorylation of Hsp27 at Ser-78 or Ser-82 has been shown to play integral roles in cellular fate (88-90). Therefore, the phosphorylation of Hsp27 at Ser-82 by PKC δ may play a yet uncharacterized role in Hsp27 regulation. Interestingly, it has been shown that dissociation of PMA-induced Hsp27 aggregates can be prevented by PKC inhibitors, while p38 inhibitors achieve a significantly smaller effect (82). Thus, PKC δ phosphorylation at Ser-82 may play an essential role in the regulation of Hsp27 through phosphorylation-induced dissociation of

Hsp27 into smaller multimers and dissociation of the molecule from a yet unidentified target at this time.

The pro-apoptotic roles of apigenin highlight the ever growing focus on chemopreventive roles of naturally-derived dietary compounds such as flavonoids. As an antioxidant, apigenin serve to regulate intracellular redox balance. However, apigenin is also potent inducer of the apoptotic pathway and the study of apigenin-induced apoptosis helps us to understand the molecular mechanisms flavonoids use to regulate cell fate and the potential use of flavonoids as chemotherapeutic agents.

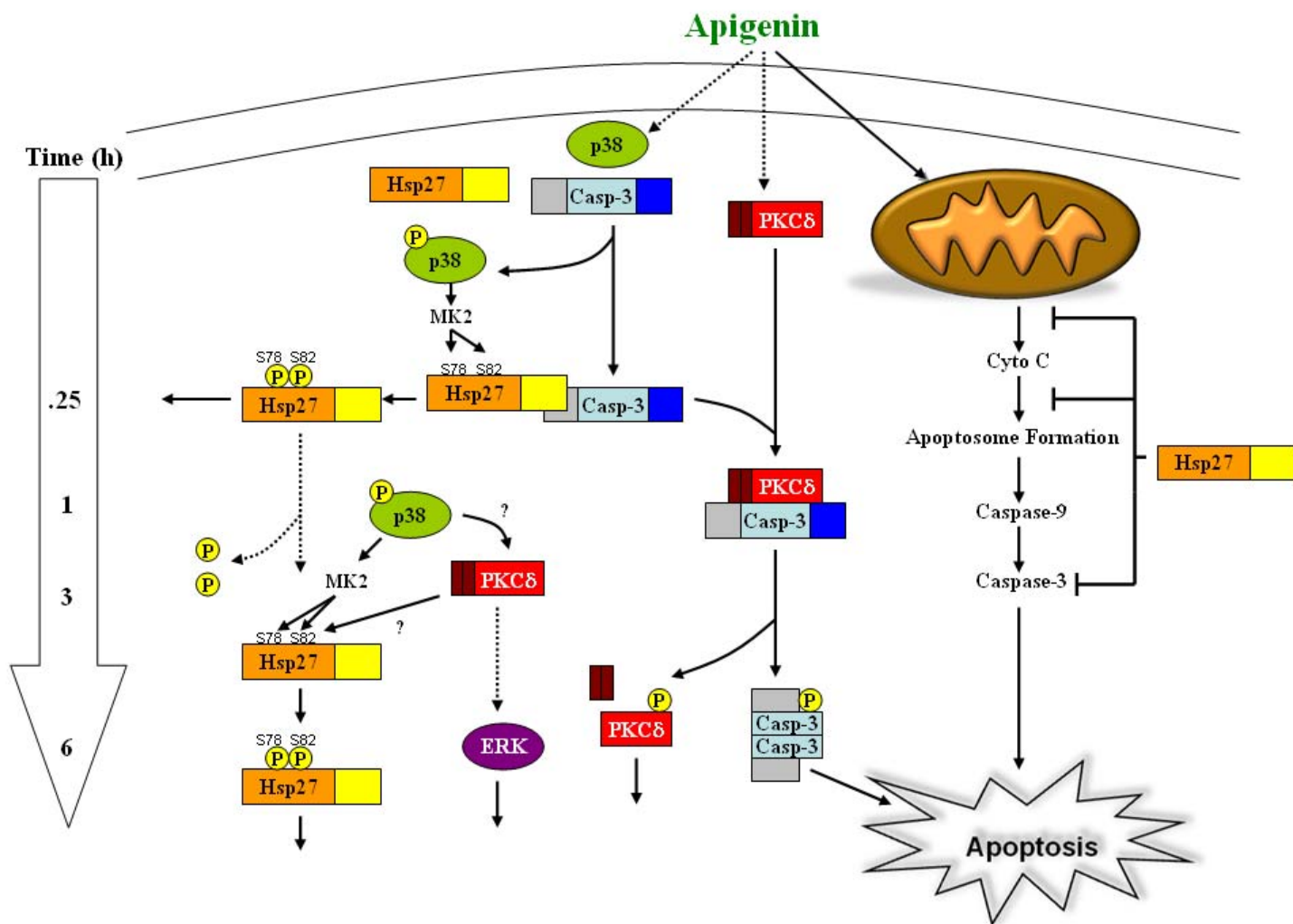


Figure 5.1 Working Model

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REFERENCES

- (1) Ellis, H. M., and Horvitz, H. R. (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44, 817-29.
- (2) Pierce, G. B., Gramzinski, R. A., and Parchment, R. E. (1989) Programmed cell death in the blastocyst. *Ann N Y Acad Sci* 567, 182-6.
- (3) Thornberry, N. A., and Lazebnik, Y. (1998) Caspases: enemies within. *Science* 281, 1312-6.
- (4) Friedlander, R. M. (2003) Apoptosis and caspases in neurodegenerative diseases. *N Engl J Med* 348, 1365-75.
- (5) Rudin, C. M., and Thompson, C. B. (1997) Apoptosis and disease: regulation and clinical relevance of programmed cell death. *Annu Rev Med* 48, 267-81.
- (6) Savill, J. (2000) Apoptosis in resolution of inflammation. *Kidney Blood Press Res* 23, 173-4.
- (7) Dive, C., and Hickman, J. A. (1991) Drug-target interactions: only the first step in the commitment to a programmed cell death? *Br J Cancer* 64, 192-6.
- (8) Hampton, M. B., Fadeel, B., and Orrenius, S. (1998) Redox regulation of the caspases during apoptosis. *Ann N Y Acad Sci* 854, 328-35.
- (9) Salvesen, G. S., and Dixit, V. M. (1997) Caspases: intracellular signaling by proteolysis. *Cell* 91, 443-6.
- (10) Adams, J. M., and Cory, S. (2002) Apoptosomes: engines for caspase activation. *Curr Opin Cell Biol* 14, 715-20.
- (11) Stennicke, H. R., and Salvesen, G. S. (1997) Biochemical characteristics of caspases-3, -6, -7, and -8. *J Biol Chem* 272, 25719-23.
- (12) Cohen, G. M. (1997) Caspases: the executioners of apoptosis. *Biochem J* 326 (Pt 1), 1-16.
- (13) Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem* 272, 17907-11.
- (14) Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) An induced proximity model for caspase-8 activation. *J Biol Chem* 273, 2926-30.
- (15) Peter, M. E., and Krammer, P. H. (1998) Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr Opin Immunol* 10, 545-51.
- (16) Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91, 479-89.
- (17) Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94, 481-90.
- (18) Saybasili, H., Yuksel, M., Haklar, G., and Yalcin, A. S. (2001) Effect of mitochondrial electron transport chain inhibitors on superoxide radical generation in rat hippocampal and striatal slices. *Antioxid Redox Signal* 3, 1099-104.

- (19) Bergamini, C. M., Gambetti, S., Dondi, A., and Cervellati, C. (2004) Oxygen, reactive oxygen species and tissue damage. *Curr Pharm Des* 10, 1611-26.
- (20) Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 144, 281-92.
- (21) Buendia, B., Santa-Maria, A., and Courvalin, J. C. (1999) Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J Cell Sci* 112 (Pt 11), 1743-53.
- (22) Rao, L., Perez, D., and White, E. (1996) Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol* 135, 1441-55.
- (23) Rudel, T., and Bokoch, G. M. (1997) Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science* 276, 1571-4.
- (24) Olson, J. M., and Hallahan, A. R. (2004) p38 MAP kinase: a convergence point in cancer therapy. *Trends Mol Med* 10, 125-9.
- (25) Bulavin, D. V., Saito, S., Hollander, M. C., Sakaguchi, K., Anderson, C. W., Appella, E., and Fornace, A. J., Jr. (1999) Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *Embo J* 18, 6845-54.
- (26) Deacon, K., Mistry, P., Chernoff, J., Blank, J. L., and Patel, R. (2003) p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. *Mol Biol Cell* 14, 2071-87.
- (27) Doseff, A. I., Baker, J. H., Jr., Bourgeois, T. A., and Wewers, M. D. (2003) Interleukin-4-induced apoptosis entails caspase activation and suppression of extracellular signal-regulated kinase phosphorylation. *Am J Respir Cell Mol Biol* 29, 367-74.
- (28) Van Dross, R., Xue, Y., Knudson, A., and Pelling, J. C. (2003) The chemopreventive bioflavonoid apigenin modulates signal transduction pathways in keratinocyte and colon carcinoma cell lines. *J Nutr* 133, 3800S-3804S.
- (29) Brodie, C., and Blumberg, P. M. (2003) Regulation of cell apoptosis by protein kinase c delta. *Apoptosis* 8, 19-27.
- (30) Voss, O. H., Kim, S., Wewers, M. D., and Doseff, A. I. (2005) Regulation of monocyte apoptosis by the protein kinase Cdelta-dependent phosphorylation of caspase-3. *J Biol Chem* 280, 17371-9.
- (31) Ghayur, T., Hugunin, M., Talanian, R. V., Ratnofsky, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. *J Exp Med* 184, 2399-404.
- (32) Reyland, M. E., Anderson, S. M., Matassa, A. A., Barzen, K. A., and Quissell, D. O. (1999) Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J Biol Chem* 274, 19115-23.

- (33) Majumder, P. K., Pandey, P., Sun, X., Cheng, K., Datta, R., Saxena, S., Kharbanda, S., and Kufe, D. (2000) Mitochondrial translocation of protein kinase C delta in phorbol ester-induced cytochrome c release and apoptosis. *J Biol Chem* 275, 21793-6.
- (34) Lee, Y. J., Lee, D. H., Cho, C. K., Chung, H. Y., Bae, S., Jhon, G. J., Soh, J. W., Jeoung, D. I., Lee, S. J., and Lee, Y. S. (2005) HSP25 inhibits radiation-induced apoptosis through reduction of PKCdelta-mediated ROS production. *Oncogene* 24, 3715-25.
- (35) Li, P. F., Maasch, C., Haller, H., Dietz, R., and von Harsdorf, R. (1999) Requirement for protein kinase C in reactive oxygen species-induced apoptosis of vascular smooth muscle cells. *Circulation* 100, 967-73.
- (36) Manach, C., Scalbert, A., Morand, C., Remesy, C., and Jimenez, L. (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79, 727-47.
- (37) Yang, C. S., Landau, J. M., Huang, M. T., and Newmark, H. L. (2001) Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr* 21, 381-406.
- (38) Le Marchand, L. (2002) Cancer preventive effects of flavonoids--a review. *Biomed Pharmacother* 56, 296-301.
- (39) Middleton, E., Jr., Kandaswami, C., and Theoharides, T. C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 52, 673-751.
- (40) Wang, H. K. (2000) The therapeutic potential of flavonoids. *Expert Opin Investig Drugs* 9, 2103-19.
- (41) Harmon, A. W., and Patel, Y. M. (2004) Naringenin inhibits glucose uptake in MCF-7 breast cancer cells: a mechanism for impaired cellular proliferation. *Breast Cancer Res Treat* 85, 103-10.
- (42) Way, T. D., Kao, M. C., and Lin, J. K. (2004) Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. *J Biol Chem* 279, 4479-89.
- (43) Wang, W., Heideman, L., Chung, C. S., Pelling, J. C., Koehler, K. J., and Birt, D. F. (2000) Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol Carcinog* 28, 102-10.
- (44) Wang, I. K., Lin-Shiau, S. Y., and Lin, J. K. (1999) Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *Eur J Cancer* 35, 1517-25.
- (45) Morrissey, C., O'Neill, A., Spengler, B., Christoffel, V., Fitzpatrick, J. M., and Watson, R. W. (2005) Apigenin drives the production of reactive oxygen species and initiates a mitochondrial mediated cell death pathway in prostate epithelial cells. *Prostate* 63, 131-42.
- (46) Miyoshi, N., Naniwa, K., Yamada, T., Osawa, T., and Nakamura, Y. (2007) Dietary flavonoid apigenin is a potential inducer of intracellular oxidative stress: the role in the interruptive apoptotic signal. *Arch Biochem Biophys* 466, 274-82.
- (47) Vargo, M. A., Voss, O. H., Poustka, F., Cardounel, A. J., Grotewold, E., and Doseff, A. I. (2006) Apigenin-induced-apoptosis is mediated by the activation of PKCdelta and caspases in leukemia cells. *Biochem Pharmacol* 72, 681-92.

- (48) Lindquist, S., and Craig, E. A. (1988) The heat-shock proteins. *Annu Rev Genet* 22, 631-77.
- (49) Cuesta, R., Laroia, G., and Schneider, R. J. (2000) Chaperone hsp27 inhibits translation during heat shock by binding eIF4G and facilitating dissociation of cap-initiation complexes. *Genes Dev* 14, 1460-70.
- (50) Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *Embo J* 16, 221-9.
- (51) Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *J Biol Chem* 268, 1517-20.
- (52) Merck, K. B., Horwitz, J., Kersten, M., Overkamp, P., Gaestel, M., Bloemendal, H., and de Jong, W. W. (1993) Comparison of the homologous carboxy-terminal domain and tail of alpha-crystallin and small heat shock protein. *Mol Biol Rep* 18, 209-15.
- (53) van Montfort, R. L., Basha, E., Friedrich, K. L., Slingsby, C., and Vierling, E. (2001) Crystal structure and assembly of a eukaryotic small heat shock protein. *Nat Struct Biol* 8, 1025-30.
- (54) Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Nohara, D., and Kato, K. (2001) Phosphorylation-induced change of the oligomerization state of alpha B-crystallin. *J Biol Chem* 276, 5346-52.
- (55) Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J., and Gaestel, M. (1999) Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem* 274, 18947-56.
- (56) MacRae, T. H. (2000) Structure and function of small heat shock/alpha-crystallin proteins: established concepts and emerging ideas. *Cell Mol Life Sci* 57, 899-913.
- (57) Paul, C., and Arrigo, A. P. (2000) Comparison of the protective activities generated by two survival proteins: Bcl-2 and Hsp27 in L929 murine fibroblasts exposed to menadione or staurosporine. *Exp Gerontol* 35, 757-66.
- (58) Lee, Y. J., Cho, H. N., Jeoung, D. I., Soh, J. W., Cho, C. K., Bae, S., Chung, H. Y., Lee, S. J., and Lee, Y. S. (2004) HSP25 overexpression attenuates oxidative stress-induced apoptosis: roles of ERK1/2 signaling and manganese superoxide dismutase. *Free Radic Biol Med* 36, 429-44.
- (59) Arrigo, A. P., Viot, S., Chaufour, S., Firdaus, W., Kretz-Remy, C., and Diaz-Latoud, C. (2005) Hsp27 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. *Antioxid Redox Signal* 7, 414-22.
- (60) Charette, S. J., Lavoie, J. N., Lambert, H., and Landry, J. (2000) Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol* 20, 7602-12.
- (61) Concannon, C. G., Orrenius, S., and Samali, A. (2001) Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. *Gene Expr* 9, 195-201.
- (62) Garrido, C., Bruey, J. M., Fromentin, A., Hammann, A., Arrigo, A. P., and Solary, E. (1999) HSP27 inhibits cytochrome c-dependent activation of procaspase-9. *Faseb J* 13, 2061-70.

- (63) Havasi, A., Li, Z., Wang, Z., Martin, J. L., Botla, V., Ruchalski, K., Schwartz, J. H., and Borkan, S. C. (2008) Hsp27 inhibits Bax activation and apoptosis via a phosphatidylinositol 3-kinase-dependent mechanism. *J Biol Chem* 283, 12305-13.
- (64) Voss, O. H., Batra, S., Kolattukudy, S. J., Gonzalez-Mejia, M. E., Smith, J. B., and Doseff, A. I. (2007) Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. *J Biol Chem* 282, 25088-99.
- (65) Bruey, J. M., Paul, C., Fromentin, A., Hilpert, S., Arrigo, A. P., Solary, E., and Garrido, C. (2000) Differential regulation of HSP27 oligomerization in tumor cells grown in vitro and in vivo. *Oncogene* 19, 4855-63.
- (66) Lee, Y. J., Lee, D. H., Cho, C. K., Bae, S., Jhon, G. J., Lee, S. J., Soh, J. W., and Lee, Y. S. (2005) HSP25 inhibits protein kinase C delta-mediated cell death through direct interaction. *J Biol Chem* 280, 18108-19.
- (67) Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., and Anderson, C. W. (1992) Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. *J Biol Chem* 267, 794-803.
- (68) Stokoe, D., Engel, K., Campbell, D. G., Cohen, P., and Gaestel, M. (1992) Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett* 313, 307-13.
- (69) Takai, S., Matsushima-Nishiwaki, R., Tokuda, H., Yasuda, E., Toyoda, H., Kaneoka, Y., Yamaguchi, A., Kumada, T., and Kozawa, O. (2007) Protein kinase C delta regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma. *Life Sci* 81, 585-91.
- (70) Maizels, E. T., Peters, C. A., Kline, M., Cutler, R. E., Jr., Shanmugam, M., and Hunzicker-Dunn, M. (1998) Heat-shock protein-25/27 phosphorylation by the delta isoform of protein kinase C. *Biochem J* 332 (Pt 3), 703-12.
- (71) Coxon, P. Y., Rane, M. J., Uriarte, S., Powell, D. W., Singh, S., Butt, W., Chen, Q., and McLeish, K. R. (2003) MAPK-activated protein kinase-2 participates in p38 MAPK-dependent and ERK-dependent functions in human neutrophils. *Cell Signal* 15, 993-1001.
- (72) Denhardt, D. T. (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. *Biochem J* 318 (Pt 3), 729-47.
- (73) Lambert, H., Charette, S. J., Bernier, A. F., Guimond, A., and Landry, J. (1999) HSP27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. *J Biol Chem* 274, 9378-85.
- (74) Zheng, C., Lin, Z., Zhao, Z. J., Yang, Y., Niu, H., and Shen, X. (2006) MAPK-activated protein kinase-2 (MK2)-mediated formation and phosphorylation-regulated dissociation of the signal complex consisting of p38, MK2, Akt, and Hsp27. *J Biol Chem* 281, 37215-26.
- (75) Landry, J., Chretien, P., Laszlo, A., and Lambert, H. (1991) Phosphorylation of HSP27 during development and decay of thermotolerance in Chinese hamster cells. *J Cell Physiol* 147, 93-101.

- (76) Raman, M., Chen, W., and Cobb, M. H. (2007) Differential regulation and properties of MAPKs. *Oncogene* 26, 3100-12.
- (77) Reyland, M. E. (2007) Protein kinase Cdelta and apoptosis. *Biochem Soc Trans* 35, 1001-4.
- (78) Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X., and Kroemer, G. (1995) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 181, 1661-72.
- (79) Geum, D., Son, G. H., and Kim, K. (2002) Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. *J Biol Chem* 277, 19913-21.
- (80) Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J., and Landry, J. (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *J Cell Sci* 110 (Pt 3), 357-68.
- (81) Chang, L., and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* 410, 37-40.
- (82) Kato, K., Ito, H., Iwamoto, I., Lida, K., and Inaguma, Y. (2001) Protein kinase inhibitors can suppress stress-induced dissociation of Hsp27. *Cell Stress Chaperones* 6, 16-20.
- (83) Jackson, R. M., and Garcia-Rojas, R. (2008) Kinase activity, heat shock protein 27 phosphorylation, and lung epithelial cell glutathione. *Exp Lung Res* 34, 245-62.
- (84) Landry, J., and Huot, J. (1995) Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. *Biochem Cell Biol* 73, 703-7.
- (85) Zucker, B., Hanusch, J., and Bauer, G. (1997) Glutathione depletion in fibroblasts is the basis for apoptosis-induction by endogenous reactive oxygen species. *Cell Death Differ* 4, 388-95.
- (86) Tang, H. L., Le, A. H., and Lung, H. L. (2006) The increase in mitochondrial association with actin precedes Bax translocation in apoptosis. *Biochem J* 396, 1-5.
- (87) Bryantsev, A. L., Kurchashova, S. Y., Golyshev, S. A., Polyakov, V. Y., Wunderink, H. F., Kanon, B., Budagova, K. R., Kabakov, A. E., and Kampinga, H. H. (2007) Regulation of stress-induced intracellular sorting and chaperone function of Hsp27 (HspB1) in mammalian cells. *Biochem J* 407, 407-17.
- (88) Rane, M. J., Pan, Y., Singh, S., Powell, D. W., Wu, R., Cummins, T., Chen, Q., McLeish, K. R., and Klein, J. B. (2003) Heat shock protein 27 controls apoptosis by regulating Akt activation. *J Biol Chem* 278, 27828-35.
- (89) Yuan, J., and Rozengurt, E. (2008) PKD, PKD2, and p38 MAPK mediate Hsp27 serine-82 phosphorylation induced by neurotensin in pancreatic cancer PANC-1 cells. *J Cell Biochem* 103, 648-62.
- (90) Niwa, M., Hotta, K., Hara, A., Hirade, K., Ito, H., Kato, K., and Kozawa, O. (2006) TNF-alpha decreases hsp 27 in human blood mononuclear cells: involvement of protein kinase c. *Life Sci* 80, 181-6.